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13. ABSTRACT ***(Maximum 200 Words)***

Germline mutations in *PTEN*, a tumor suppressor gene located on 10q23.3, is associated with 80% of Cowden syndrome (CS) probands ascertained by the strict operational diagnostic criteria of the International Cowden Consortium. CS is an inherited multiple hamartoma syndrome with a high risk of breast and thyroid cancer. The PI has found that ~5% of unselected apparently sporadic breast cancer patients diagnosed under the age of 40 carry occult germline *PTEN* mutations. Therefore, this DAMD project sought to determine if *PTEN* played a role in non-*BRCA/2* familial site-specific breast cancer and patients and families who have partial features of CS but who do not meet full Consortium criteria. To date, the PI has found that germline *PTEN* mutations do not play a role in familial site-specific breast cancer. However, CS-like individuals and families have at least a 5% probability of carrying germline *PTEN* mutations, with full implications for cancer risk and surveillance for that individual and the family. The presence of endometrial cancer seems to increase the likelihood of finding a *PTEN* mutation in a CS-like family or case. These findings have helped revise the Consortium diagnostic criteria which will be adopted into the NCCN guidelines for their next version. Further, because of the apparent importance of endometrial carcinoma, despite its relatively low frequency, in CS, the PI extended her work to examine *PTEN* mutation and expression in sporadic endometrial cancer and precancers and discovered that at least 93% of all endometrial carcinomas are due to *PTEN* mutation or epigenetic PTEN silencing, and that PTEN alterations already are present in a subset of the earliest endometrial precancers.

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PI: Charis Eng, MD, PhD
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INTRODUCTION

Germline mutations in *PTEN*, encoding a dual specificity phosphatase tumor suppressor on 10q23.3, are associated with 80% of Cowden syndrome (CS) cases ascertained by the criteria of the International Cowden Consortium (1, 2). CS is an autosomal dominant disorder characterized by multiple hamartomas and a high risk of breast and thyroid cancers. Further, approximately 60% of Bannayan-Riley-Ruvalcaba syndrome cases (BRR) have germline *PTEN* mutations as well, thus making CS and BRR allelic (3). Initially felt to be unrelated, BRR is characterized by macrocephaly, lipomatosis, and speckled penis, and until the PI's analyses, was felt not to be associated with cancer. Subsequently, a non-BRR, non-CS, Proteus-like individual was found to have a germline and a germline mosaic *PTEN* mutation (4). The PI had proposed to determine whether and at what frequency occult germline *PTEN* mutations occur in non-CS/BRR families. Towards these ends, the specific aims were:

1. To determine the frequency and nature of germline *PTEN* mutations in non-CS/BRR site-specific breast cancer families; and
2. To determine the frequency and nature of germline *PTEN* mutations in non-CS/BRR breast-thyroid and/or endometrial carcinoma families/individuals, so called CS-like cases.

BODY

Task 1: Mutation Analysis in Non-CS Breast Cancer Families

Up through Year 1, 15 *BRCA1/2* negative site specific familial breast cancer cases were ascertained. In Year 2, another 6 were accrued, and amongst all 21 were *PTEN* mutation negative and where informative, were not linked to 10q. In view of these findings and two recent published reports (5, 6), we know that site specific breast cancer families are not accounted for by *PTEN* mutation. Because of this overwhelming data, the PI will truncate accrual of this sub-project. Instead, in view of the interesting data obtained from Task 2 (below), the PI will focus the major efforts of Year 3 on Task 2, and tie Task 1 to Task 2 by accrual of non-CS families segregating breast and endometrial cancers or single individuals with both breast and endometrial cancers but who do not meet the diagnostic criteria of CS.

Following up on plans noted in the PI's first Annual Report, the PI has examined a series of well characterized sporadic breast carcinomas to correlate *PTEN* somatic mutation and PTEN expression by immunohistochemistry using a specific monoclonal antibody 6H2.1 (7). These observations are detailed in a reprint enclosed in the Appendix (7). In brief, the PI has found that somatic intragenic *PTEN* mutations rarely occur in primary breast carcinomas. Instead, hemizygous deletion as well as epigenetic silencing are the mechanisms of inactivation of PTEN in breast carcinogenesis and they pertain in approximately 15-40% of all primary sporadic adenocarcinomas of the breast. These

observations will be followed up by determining the precise mechanism of epigenetic silencing in Year 3 and thereafter.

Task 2: Mutation Analysis in Non-CS Breast-Thyroid and/or Endometrial Carcinoma Families/Individuals ("CS-Like Families")

In Year 1 of this grant, the PI reported on a study to examine germline *PTEN* mutations in families and individuals ascertained by the minimal presence of breast cancer and any anatomical thyroid disorder in a single individual or in a minimum of two first-degree relatives in a family but who did not meet the Consortium criteria for the diagnosis of CS (8). Of 64 CS-like cases ascertained, one was found to have a germline *PTEN* mutation. This family had bilateral breast cancer, follicular thyroid carcinoma and endometrial adenocarcinoma. There were only 4 other families with endometrial cancer. In the past year (Year 2 of the grant), the PI has continued to accrue CS-like families with endometrial carcinoma, usually breast and endometrium occurring in a minimum of two related individuals or in a single individual, as well as CS-like families with breast cancer, any structural thyroid disorder and endometrial carcinoma in a minimum of two related individuals or in a single individual. To date, 7 more unrelated probands have been accrued, 6 have been mutation analyzed and 2 have been found to carry germline *PTEN* mutations.

Because of these studies examining CS-like families (8), especially those containing endometrial carcinomas, the PI's data suggests that the presence of endometrial cancer may increase the likelihood of finding germline *PTEN* mutation, even in CS-like families. In another recent study, a nested cohort comprising 103 eligible women with multiple primary cancers within the 32 826-member Nurses' Health Study were examined for the occult presence of germline *PTEN* mutations (9). Among 103 cases, 5 (5%) were found to have germline missense mutations, all of which have been shown to cause some loss-of-function. Of these 5, 2 cases themselves had endometrial cancer. This study, therefore, suggests that occult germline mutations of *PTEN* and by extrapolation, CS, occur with a higher frequency than previously believed. Further, these data confirm the PI's previous observations (8) that endometrial carcinoma might be an important component cancer of CS, and indeed, its presence in a case or family that is reminiscent of CS but does not meet Consortium criteria might actually help increase the prior probablity of finding *PTEN* mutation. Taken together, these molecular-based observations, together with previous clinical epidemiologic studies, (10) were felt sufficient to revise the Consortium criteria for the diagnosis of CS to include endometrial carcinoma (Table 1) (11). The inclusion of endometrial carcinoma to the Consortium operational diagnostic criteria will most likely be adopted by the National Comprehensive Cancer Center Genetics/High Risk Panel at its next revision.

Table 1. International Cowden Consortium operational criteria for the diagnosis of CS, Ver. 2000

<u>Pathognomonic Criteria</u>

Mucocutanous lesions:

- Trichilemmomas, facial
- Acral keratoses

Papillomatous papules
Mucosal lesions

Major Criteria
Breast carcinoma
Thyroid carcinoma (non-medullary), esp. follicular thyroid carcinoma
Macrocephaly (Megalencephaly) (say, ≥95%ile)
Lhermitte-Duclos disease (LDD)
Endometrial carcinoma
Minor Criteria
Other thyroid lesions (e.g adenoma or multinodular goiter)
Mental retardation (say, IQ ≤75)
GI hamartomas
Fibrocystic disease of the breast
Lipomas
Fibromas
GU tumors (eg, **renal cell carcinoma**, uterine fibroids) or malformation

Operational Diagnosis in an Individual:
1. Mucocutanous lesions alone if:
a) there are 6 or more facial papules, of which 3 or more must be trichilemmoma, or
b) cutaneous facial papules and oral mucosal papillomatosis, or
c) oral mucosal papillomatosis and acral keratoses, or
d) palmo plantar keratoses, 6 or more
2. 2 Major criteria but one must include macrocephaly or LDD
3. 1 Major and 3 minor criteria
4. 4 minor criteria

Operational Diagnosis in a Family where One Individual is Diagnostic for Cowden
1. The pathognomonic criterion/ia
2. Any one major criterion with or without minor criteria
3. Two minor criteria

*Operational diagnostic criteria are reviewed and revised on a continuous basis as new clinical and genetic information becomes available.

Because endometrial carcinoma occurrence per se in classic CS individuals and families are not as frequent as breast or thyroid carcinomas and yet appears to be a very important determinant of *PTEN* germline status, the PI then sought to examine the normal cycling endometrium and sporadic endometrial cancers and precancers as they relate to *PTEN* mutation and expression. As further detailed in an accompanying reprint in the Appendix (12), the PI and collaborator found that under estrogenic predominance, the proliferative endometrium shows ubiquitous nuclear and cytoplasmic PTEN expression by immunohistochemistry with a specific monoclonal antibody 6H2.1 (7). After 3-4 days of progestational exposure (primed with estrogen), the glandular epithelium maintains cytoplasmic PTEN expression, with waning nuclear expression (12). These observations

suggest that PTEN expression might be modulated by sex steroid hormones that may have implications for neoplasia not only of the endometrium but also of the breast.

When the PI and collaborator investigated neoplastic endometria in relationship to *PTEN* mutation and expression, they found both mutation and loss of expression occurred even in the earliest endometrial precancers (13). These observations are detailed in an accompanying reprint (13) found in the Appendix. The data suggest that loss of PTEN expression can precede intragenic *PTEN* mutation even in the endometrial precancer stage and that either *PTEN* mutation or epigenetic PTEN silencing together play a role in >90% of sporadic endometrial carcinomas.

KEY RESEARCH ACCOMPLISHMENTS

- That endometrial carcinoma occurring in a CS-like individual or family might increase the prior probability of finding a germline *PTEN* mutation.
- Because of the PI's observations, the Operational Diagnostic Criteria of the International Cowden Consortium has been revised to include endometrial carcinoma as a major component criterion.
- The revised Consortium criteria will likely be adopted into the next revision of the National Comprehensive Cancer Network Genetics/High Risk Panel recommendations.
- That PTEN plays a major role in sporadic endometrial precancer and cancer.
- That PTEN plays an important role in sporadic breast carcinogenesis, accounting for perhaps 15-40% of all such cancers.

REPORTABLE OUTCOMES

Manuscripts, Abstracts and Presentations

Perren A, Weng LP, Boag AH, Ziebold U, Kum JB, Dahia PLM, Komminoth P, Lees JA, Mulligan LM, Mutter GL, **Eng C**. Immunocytochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol 1999; 155:1253-60.

Eng C. Commentary. Will the real Cowden syndrome please stand up: new diagnostic criteria. J Med Genet (in press)

Mutter GL, Lin M-C, FitzGerald JT, Kum JB, **Eng C**. Changes in endometrial *PTEN* expression throughout the human menstrual cycle. J Clin Endocrinol Metab 2000; 85:2334-8.

Mutter GL, Lin M-C, FitzGerald JT, Kum JB, Baak JPA, Lees JA, Weng LP, **Eng C**. Altered *PTEN* expression as a molecular diagnostic marker for the earliest endometrial precancers. J Natl Cancer Inst 2000; 92:924-31.

Informatics, Databases, Etc

Continued expansion of clinical cancer genetics CS/BRR/CS-like clinical-genotype database.

Funding Applied For Partially Based on Work Funded by This Award

National Institutes of Health R01 (Oct. 1, 2000 deadline) entitled, "Individual and Age-Dependent Risk of Cancer in *PTEN* Syndromes"

American Cancer Society Research Scholar Grant (Oct. 15, 2000 deadline) entitled, "Genetics of *PTEN* in Cowden and Related Syndromes and Familial Breast Cancer"

CONCLUSIONS

To date, because of work related to Task 1, we know that non-syndromic, site-specific familial breast cancer that is not associated with *BRCA1* and *BRCA2* is not associated with germline *PTEN* mutations. Our findings have been corroborated by two other groups (5, 6). This is an instructive negative finding in light of the fact that the PI had also found (unrelated to this DAMD award) that approximately 5% of unselected, apparently isolated breast cancer presentations before the age of 40 carry occult germline *PTEN* mutations (14). Further, the PI has uncovered epigenetic phenomena related to PTEN silencing in a proportion of sporadic breast adenocarcinomas.

Work related to Task 2 has yielded interesting data, which have important implications for the practice of clinical cancer genetics. Individuals or families who have some but not all features of CS, ascertained in a specific way (noted above), have a minimum 5% probability of carrying germline *PTEN* mutations. The probability of having a mutation might be increased with the presence of endometrial carcinoma either in the proband or a relative. These findings have led to a revision of the diagnostic criteria of the International Cowden Consortium as well as the National Comprehensive Cancer Network. Because of the finding that endometrial carcinoma might be a key feature indicating CS, the PI explored sporadic endometrial carcinoma and has shown that PTEN plays a major role in sporadic endometrial carcinogenesis as well.

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APPENDIX

1. REPRINTS/PRE-PRINTS

Perren A, Weng LP, Boag AH, Ziebold U, Kum JB, Dahia PLM, Komminoth P, Lees JA, Mulligan LM, Mutter GL, **Eng C**. Immunocytochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol 1999; 155:1253-60.

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2. PI'S CURRICULUM VITAE

American Journal of Pathology, Vol. 155, No. 4, October 1999

Immunohistochemical Evidence of Loss of PTEN Expression in Primary Ductal Adenocarcinomas of the Breast

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Germline mutations in *PTEN*, encoding a dual-specificity phosphatase on 10q23.3, cause Cowden syndrome (CS), which is characterized by a high risk of breast and thyroid cancers. Loss of heterozygosity of 10q22–24 markers and somatic *PTEN* mutations have been found to a greater or lesser extent in a variety of sporadic component and noncomponent cancers of CS. Among several series of sporadic breast carcinomas, the frequency of loss of flanking markers around *PTEN* is approximately 30 to 40%, and the somatic intragenic *PTEN* mutation frequency is <5%. In this study, we analyzed PTEN expression in 33 sporadic primary breast carcinoma samples using immunohistochemistry and correlated this to structural studies at the molecular level. Normal mammary tissue had a distinctive pattern of expression: myoepithelial cells uniformly showed strong PTEN expression. The PTEN protein level in mammary epithelial cells was variable. Ductal hyperplasia with and without atypia exhibited higher PTEN protein levels than normal mammary epithelial cells. Among the 33 carcinoma samples, 5 (15%) were immunohistochemically PTEN-negative; 6 (18%) had reduced staining, and the rest were PTEN-positive. In the PTEN-positive tumors as well as in normal epithelium, the protein was localized in the cytoplasm and in the nucleus (or nuclear membrane). Among the immunostain negative group, all had hemizygous *PTEN* deletion but no structural alteration of the remaining allele. Thus, in these cases, an epigenetic phenomenon such as hypermethylation, decreased protein synthesis or increased protein degradation may be involved. In the cases with reduced staining, 5 of 6 had hemizygous *PTEN* deletion and 1 did not have any structural abnormality. Finally, clinicopathological features were analyzed against PTEN protein expression. Three of the 5 PTEN immunostain-negative carcinomas were also both estrogen and progesterone receptor-negative, whereas only 5 of 22 of the PTEN-positive group were double receptor-negative. The significance of this last observation requires further study. *(Am J Pathol 1999, 155:1253–1260)*

The tumor suppressor gene *PTEN*, encoding a dual-specificity phosphatase, has been cloned and mapped to chromosome 10q23.3.[1–3] Germline *PTEN* mutations are found in the autosomal dominant Cowden syndrome (CS), which is characterized by multiple hamartomas involving many organ systems as well as an increased risk of developing breast and thyroid cancers.[4,5] Loss of heterozygosity (LOH) of markers at 10q23–25 is a frequent event (30–50%) in endometrial cancer,[6–9] glioblastoma,[10] and breast cancer.[11–13] Somatic intragenic mutations of *PTEN* are a frequent event in endometrial carcinomas,[6–9] malignant gliomas,[14–17] and melanomas.[18] However, unlike endometrial carcinoma and glioblastoma, only a very small fraction (<5%) of the 40% of primary breast cancers showing allelic loss in this region also have mutations in the remaining allele,[11–13,19] de-

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spite the fact that females with CS have a ≤50% lifetime risk of developing breast cancer.[5,20,21] In contrast to these analyses based on primary breast carcinomas, initial studies using breast cancer cell lines seemed to show that a large proportion have biallelic loss of *PTEN*.[1,3] Investigators, therefore, questioned whether loss of one *PTEN* allele (haploinsufficiency) is sufficient for tumorigenesis or whether inactivation of the second allele might occur through epigenetic rather than mutational events.

We report a study of PTEN expression using immunohistochemical methods in a series of 33 primary human breast tumors. This is a powerful method because it provides an internal control comparing the staining of tumor tissue to that of the adjacent normal breast tissue. We also began to explore the association of *PTEN* expression with genomic *PTEN* status and clinicopathological features.

Materials and Methods

Breast Carcinoma Samples

Paraffin blocks of 33 unselected sporadic primary ductal breast carcinomas were drawn from the files of the Kingston General Hospital (Kingston, ON, Canada). LOH analysis with seven microsatellite markers known to map to the 10q23 interval and flanking *PTEN* as well as *PTEN* mutation analysis have been performed previously.[13] Of the 33 women diagnosed with primary mammary adenocarcinomas, 4 were diagnosed before the age of 50. The tumors ranged in size from 1 to 6 cm. There were 2 well differentiated, 13 moderately differentiated, and 18 poorly differentiated tumors. Seven of the 13 women had regional lymph node involvement at presentation.

Immunohistochemistry

The monoclonal antibody 6H2.1 raised against the last 100 C-terminal amino acids of PTEN (Ziebold and Lees, unpublished) was used in all immunohistochemical analyses.

The tissue samples were fixed by immersion in 10% buffered formalin and embedded in paraffin according to standard procedures.[22] Four-millimeter sections were cut and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Immunostaining was performed essentially as described.[22–24] In summary, the sections were deparaffinized and hydrated by passing through xylene and a graded series of ethanol. Antigen retrieval was performed for 20 minutes at 98°C in 0.01 mol/L sodium citrate buffer, pH 6.4, in a microwave oven. Incubating the sections in 0.3% hydrogen peroxide for 30 minutes blocked endogenous peroxidase activity. After blocking for 30 minutes in 0.75% normal horse serum the sections were incubated with 6H2.1 (dilution 1:100) for 1 hour at room temperature. The sections were washed in Tris-buffered saline and then incubated with biotinylated horse anti-mouse IgG followed by avidin peroxidase using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA). The chromogenic reaction was carried out with 3–3′ diaminobenzidine using nickel cobalt amplification,[25] which gives a black product. After counterstaining with Nuclear Fast Red (Rowley Biochemical, Danvers, MA) and mounting, the slides were evaluated under a light microscope. According to the amount of staining, the tumors were divided in three groups: the group assigned ++ showed increased or equal staining intensity compared to the corresponding normal tissue; the group assigned + had decreased staining intensity; and the group assigned − had no trace of staining.

A series of commercially available cell lines with known PTEN status was used as positive and negative controls to prove antibody specificity by immunohistochemistry: Balb C/3T3, Nalm6, DU145, MDA-MB-468, A172, and PC3 (see Results). In addition, the U2OS osteosarcoma cell line was transfected with full length *PTEN* cDNA expression construct as a further positive control.

Incubating the sections in the absence of antibody as well as preincubation during 2 hours at 37°C of the antibody with recombinant PTEN protein led to negative results (data not shown).

Western Blot Analysis

As biochemical proof of antibody specificity for PTEN, total protein lysates were obtained[26] (Dahia, 1999 #955) from a series of commercially available cell lines (American Type Culture Collection, Manassas, VA), for which PTEN status is known: MCF-7, T47D, MDA-MB-435S, ZR-75-1, BT-549, and MDA-MB-468 (see Results). In addition, as an additional positive control, the wild-type full length human PTEN cDNA sequence was cloned into the mammalian expression vector pUHD10-3, which contains a tetracycline-suppressible promoter (Gossen, 1992 #1065), and stably transfected into the MCF7/T-off (Clontech, Palo Alto, CA) breast cancer cell line. After 24 hours of tetracycline withdrawal for purposes of PTEN induction, protein lysates were collected for Western blot analysis as well. Western blot analysis was performed as previously described,[26] except that 6H2.1 was used at a 1:250 dilution. Control antibody was against α-tubulin and used at 1:1000 dilution.

LOH Analysis

All breast carcinoma samples have been previously evaluated for LOH with markers closely flanking, but not within, *PTEN*.[13] In the event that our immunohistochemical results seemed to be discordant with the molecular analyses, further LOH analysis was performed using markers within *PTEN* itself as previously described.[26,27] Potential hemizygosity at the *PTEN* locus was assessed by screening for a T/G polymorphism within *PTEN* intron 8 detected by differential digestion with the restriction endonuclease *Hin*cII as previously described[27] and the intragenic markers D10S2491, AFM086wg9, and D10S2492.

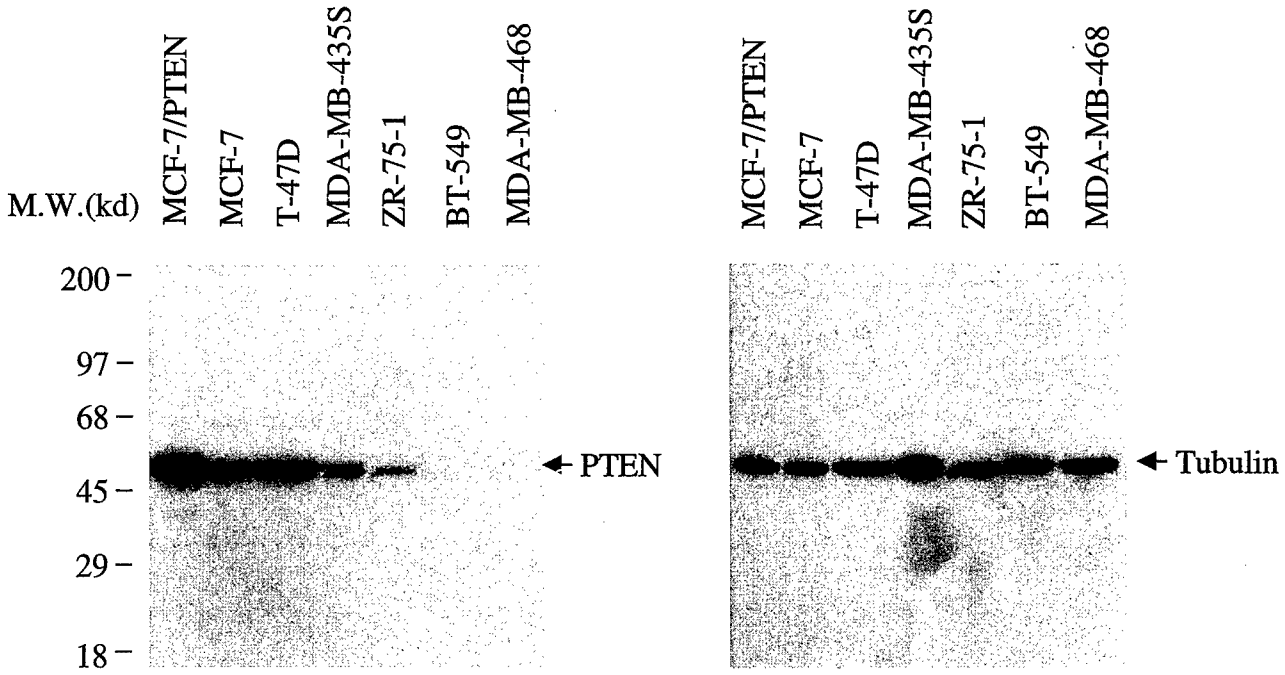

Figure 1. Western blot of 7 breast cancer cell lines using the anti-PTEN monoclonal antibody 6H2.1 (**left panel**) and using the anti-α-tubulin antibody as a control (**right panel**). MCF-7, T-47D, and MDA-MB-435S have endogenous PTEN. BT-549 and MDA-MB-468 are PTEN-null. ZR-75-1 has monoallelic PTEN deletion and a missense mutation on the remaining allele. MCF-7/PTEN is the MCF-7 line transfected with a wild-type PTEN construct and a tetracycline-inducible promoter after withdrawal of tetracycline and, hence, induced expression of PTEN.

Results

Specificity of Monoclonal Antibody 6H2.1

Because this study relied on a monoclonal antibody, 6H2.1, specific recognition of PTEN by this antibody is crucial. Western blot analysis using a series of breast cancer lines with known PTEN status and the 6H2.1 anti-PTEN monoclonal antibody demonstrated the specificity of this antibody (Figure 1). Western analysis of three PTEN+/+ lines, MCF-7, T-47D, and MDA-MB-435S, revealed a single band at the molecular weight predicted for PTEN. After induction of MCF-7/PTEN, increased expression of PTEN was evidenced by an increased band intensity (Figure 1). In contrast, ZR-75-1, with a hemizygous deletion of *PTEN* and a missense mutation in the remaining allele, yielded a weak band of the expected size. BT-549 and MDA-MB-468, which are null for PTEN, had no signal. No nonspecific bands were noted. Control blot with anti-α-tubulin antibody revealed signals for all lines.

To test the suitability of the antibody for immunohistochemistry, we used PTEN-transfected U2OS cells as well as a series of cell lines expressing PTEN (Balb C/3T3, Nalm6, DU145) as positive controls. MDA-MB-468, a breast cancer cell line with a hemizygous deletion of PTEN and a truncating mutation of the remaining allele, A172, a glioblastoma cell line with loss of one PTEN allele and a truncating mutation in exon 2 of the remaining allele and PC3, a prostate cell line with homozygous deletion of PTEN, were used as negative controls (data not shown).

PTEN Immunohistochemistry in Primary Breast Carcinomas

Samples from 33 sporadic primary breast carcinomas, which had been examined previously for LOH of markers flanking *PTEN* as well as somatic *PTEN* mutations,[13] were subjected to immunohistochemical analysis using a monoclonal antibody, 6H2.1, raised against the terminal 100 amino acids of human PTEN. Of the 33 total cancers, 29 had accompanying normal tissue; in each of the 29 samples, the normal glandular epithelium showed immunoreactivity to 6H2.1. Interestingly, there was a distinctive staining pattern in the normal tissue. The myoepithelial cells of the normal ducts showed the strongest signal with a nuclear predominance (Figure 2B). In contrast, the amount of staining in the epithelial cell layer was variable. Areas of epithelial ductal hyperplasia with and without atypia stained more strongly than the normal epithelia (Figure 2A). Endothelial cells, especially within neovascular capillaries, and nerves showed strong PTEN expression and were useful as internal positive controls.

Of 33 breast carcinoma samples, 5 (15%) lost all PTEN immunoreactivity and showed negative immunostaining, graded − (Table 1 and Figure 2, E and F). In each of these 5 cases, adjacent non-neoplastic glands (Figure 2F) as well as enclosed non-neoplastic ducts (Figure 2 E) stained positively. Interestingly, the cells within the desmoplastic reaction surrounding each of these 5 carcinomas had high PTEN expression. Six of the 33 (18%) breast cancer specimens stained weakly, graded +, in

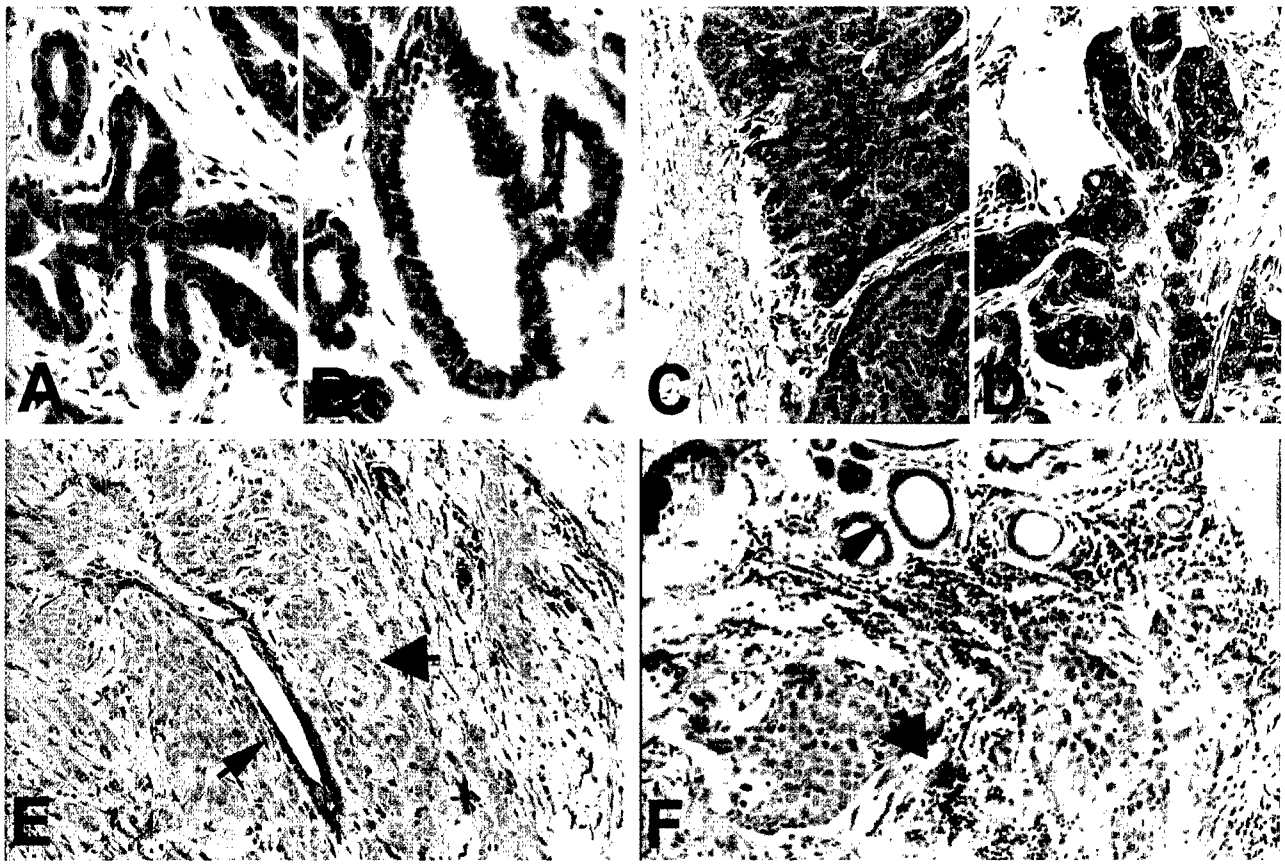

Figure 2. **A**: Ductal hyperplasia (case 58) with increased staining in the epithelial layer (original magnification, ×60). **B**: Normal breast glands (case 46) with predominantly nuclear staining in the myoepithelial layer (original magnification, ×60). **C** (case 48) and **D** (case 43): Ductal carcinoma with strong PTEN staining (++, original magnification, ×30). **E**: Ductal PTEN-negative carcinoma (**arrowhead**, case 58) and surrounding normal duct (**arrow**). Original magnification, ×30. **F**: Ductal PTEN-negative carcinoma (**arrowhead**, case 46) with non-neoplastic normal duct (**arrow**). Original magnification, ×30.

comparison to the normal tissue (Table 1 and Figure 3). One of these tumors (Sample 40, Table 1) showed positive immunostaining in the intraductal component, whereas the adjacent invasive component lost almost all PTEN protein expression (Figure 3A). The remaining 22 (66%) tumors stained positively, graded ++ (increased staining compared to normal glands). All these tumors showed homogeneous PTEN immunoreactivity throughout the examined section. PTEN immunoreactivity in these 22 tumors as well as their corresponding normal and hyperplastic breast tissue involved the cytoplasmic and nuclear (most likely nuclear membrane) compartment of the cells.

Table 1. Correlation between PTEN Immunostaining and *PTEN* and/or 10q22-23 LOH

	PTEN Immuno ++	PTEN Immuno +	PTEN Immuno −
LOH 5′ Markers	4*	2	4
LOH 3′ Markers	1*	2	5
ROH Flank Markers	18	2*	0
Total Tumors	22	6	5

Correlation between PTEN immunostaining and LOH of 5′ and/or 3′ flanking markers.
Concordance 82%.
LOH, loss of heterozygosity; ROH, retention of heterozygosity.
*Apparent discordance 18%.

Comparison of Immunohistochemical and Structural Mutation Data

Immunohistochemical evidence of PTEN expression was absent or weak in a total of 11 (33%) of 33 breast carcinomas. These breast carcinomas had been previously examined for LOH of markers flanking *PTEN* and also for intragenic *PTEN* mutations;[13] 40% demonstrated LOH but there were no intragenic *PTEN* mutations or biallelic deletion. Whether there is a one-to-one concordance between molecular and immunohistochemical observations is further explored in this report.

LOH analysis for markers in the 10q22–24 interval was previously performed using seven microsatellite markers (centromeric to telomeric): D10S579, D10S215, D10S1765, D10S541, D10S1735, D10S1739, and D10S564.[13] *PTEN* lies between D10S1765 and D10S541, a genetic distance of 1 cM but a physical distance of only several hundred kilobasepairs. For purposes of this study, to compare the immunohistochemical data to the LOH data, *PTEN* was considered to be physically deleted only when one or more immediately flanking (informative) markers centromeric and telomeric of *PTEN* showed LOH. Using this strict and conservative interpretation for monoallelic *PTEN* deletion, 6 of the tumors were shown to have a loss of one allele of the *PTEN* gene, another 7 were shown to have a loss flanking one side of (which may or

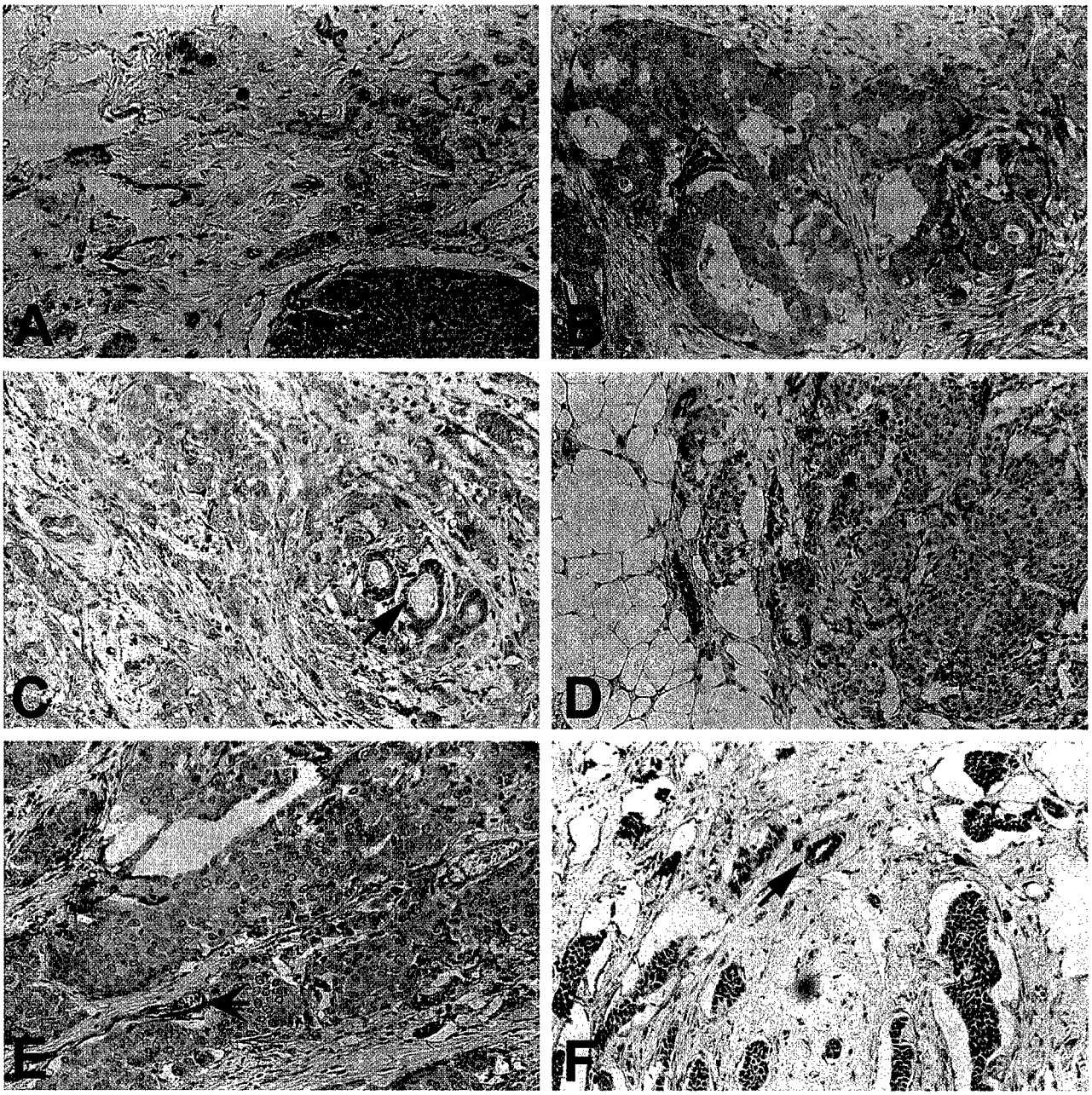

Figure 3. Cases with weak staining (**arrows** in **C** and **F**, non-neoplastic duct; **arrow** in **E**, blood vessel). **A**: Ductal carcinoma (case 40) showing no staining (graded −) in the invasive component (**top**) adjacent to immunostain-positive intraductal component (**bottom**). **B**: Case 66. **C**: Case 59. **D**: Case 57. **E**: Case 45. Original magnification, ×30.

may not include) *PTEN*. For these latter 7 tumors, potential hemizygosity at the *PTEN* locus was further assessed by screening for a T/G polymorphism within *PTEN* intron 8 (IVS8+32T/G), detected by differential digestion with the restriction endonuclease *Hin*cII, and the intragenic polymorphic markers AFM086wg9, D10S2491, and D10S2492. AFM086wg9 lies in intron 2 of *PTEN*. The likely intragenic marker order is centromere – D10S2491 – AFM086wg9 – D10S2492/IVS8+32T/G – telomere (Marsh and Eng, unpublished).

Of the 5 breast carcinomas that exhibited no immunohistochemical evidence of PTEN expression (graded −), 4 showed extensive LOH of markers flanking *PTEN* and hence, *PTEN* itself (Table 1, Column 3 and Table 3). The fifth carcinoma had LOH on the telomeric side (D10S541) of *PTEN*. Further molecular analysis revealed retention of heterozygosity at AFM086wg9 but LOH at the IVS8+32T/G polymorphism, suggesting hemizygous deletion of the 3′ end of *PTEN*. Therefore, all 5 breast carcinomas that had negative PTEN immunostaining also had hemizygous *PTEN* deletion (Table 3). None of these 5 had biallelic deletion of *PTEN* nor did they have a second intragenic *PTEN* hit, ie, mutation of the remaining allele.

Of the 6 carcinomas that had weak PTEN immunostaining, graded +, 4 had been previously shown to have LOH of markers flanking one side or the other of *PTEN* and 2

Table 2. Analysis of Correlation between PTEN Immunostaining and PTEN Intragenic LOH in Cases with Decreased Immunostaining and Apparently Discordant Tumors

Tumor	Immuno-staining score	PTEN 10q22-23 Markers S1765	S2491	AFM086	S2492	IVS8	S541
41	++	LOH	NI	LOH	ROH	ROH	NI
52	++	LOH	NI	ROH	ROH	N/A	ROH
53	++	LOH	ROH	ROH	ROH	ROH	LOH
50	++	LOH	ROH	ROH	NI	ROH	NI
40	+	LOH	N/A	NI	N/A	LOH	ROH
59	+	LOH	N/A	LOH	N/A	LOH	ROH
66	+	ROH	NI	LOH	N/A	N/A	LOH
57	+	ROH	LOH	LOH	N/A	N/A	ROH
55	+	ROH	NI	NI	N/A	LOH	LOH
45	+	ROH	NI	ROH	NI	ROH	ROH

Tumor numbers correspond to those of Feilotter et al.[13]
LOH, loss of heterozygosity; ROH, retention of heterozygosity; NI, not informative (germline homozygosity at marker); N/A, not applicable or not done.

showed no LOH of flanking markers (Tables 2 and 3). Further LOH analysis within *PTEN* revealed that the 4 carcinomas with LOH of markers flanking one side of the gene also had LOH of at least one of the intragenic markers (Table 2). Thus, these 4 tumors with decreased immunostaining seemed to have hemizygous deletion of *PTEN* or at least part of it. In the remaining two carcinomas without LOH of markers immediately flanking the gene, further analyses within the gene were uninformative or showed retention of heterozygosity (Tumors 45 and 57, Tables 2 and 3). In all likelihood, *PTEN* might not be altered at the structural level in that particular tumor.

Among the remaining 22 carcinomas that showed immunohistochemical evidence of strong PTEN expression (increased staining compared to normal mammary glands), 18 (82%) showed no LOH and biallelic presence of *PTEN* was demonstrated (Table 1). There were 4 tumors that seemed to be immunostained (grade ++), yet showed LOH flanking *PTEN* (Tables 2 and 3). However, it should be noted that 3 of these 4 tumors had LOH of D10S1765 immediately centromeric of *PTEN* but with either retention of heterozygosity or noninformativeness at D10S541 immediately 3′ of the gene. Further LOH analysis within *PTEN* corroborates the previous observations (Table 2): in tumor 6, 3′ markers within the gene showed retention of heterozygosity and a 5′marker (AFM086wg9) showed LOH; in tumor 5, where D10S1765 showed LOH, markers within the gene (AFM086wg9 and D10S2492) and 3′ of the gene (D10S541) all showed retention of heterozygosity. Similarly, tumor 9, which had LOH at D10S1765, had 3 of 4 intragenic markers with retention of heterozygosity. Tumor 53 was unusual in that both D10S1765 and D10S541 had LOH, although molecular analysis demonstrated all 4 intragenic markers with retained heterozygosity.

Table 3. Summary of PTEN Expression by Immunohistochemistry Compared to Molecular Analysis

PTEN Expression	LOH*	ROH
PTEN−	5	0
PTEN+	5	1
PTEN++	1	21

*LOH of both flanking markers or a minimum of LOH of one intragenic marker.

Table 4. Estrogen/Progesterone Receptor Status of Breast Carcinomas by PTEN Immunostaining Status

PTEN IHC status	ER/PR −	ER/PR +
Negative (−)	3	2
Decreased (+)	0	7
Positive (++)	5	16

ER, estrogen receptor; PR, progesterone receptor.
An equivocal positive receptor status (n = 5) was scored as a positive.

Correlation of PTEN Immunohistochemistry and Clinicopathological Parameters

PTEN immunostaining status was compared with such clinicopathological parameters as age at diagnosis, size of primary tumor, tumor grade, lymph node status, and estrogen receptor and progesterone receptor status. Because of the relatively small numbers, especially in the context of subset analyses, no conclusions could be drawn with confidence from our observed correlations. The most interesting association seemed to be that between PTEN expression and hormone receptor status (Table 4). Three of the 5 carcinomas (67%) that had no PTEN protein were estrogen and progesterone receptor-negative compared to 5 of 22 (23%; $P < 0.05$ Fisher's exact test) in the PTEN-immunopositive samples. All 6 carcinomas that had weak PTEN staining were estrogen and progesterone receptor-positive. Other trends are also noteworthy. Although there were only 2 grade I tumors, both had high PTEN expression. All 5 tumors that were 1.5 cm or smaller had high levels of PTEN protein.

Discussion

In this first report of immunohistochemical analyses of PTEN expression in sporadic primary breast carcinomas, we found that 33% of these tumors had either no or decreased expression of PTEN, which generally appeared to correlate with structural monoallelic deletion of the gene. Although it is understandable that tumors with monoallelic loss of *PTEN* have decreased PTEN expression at the protein level, one must explain the 5 samples with no immunoreactivity and structural *PTEN* hemizygosity. None of these samples was found to have intragenic *PTEN* mutations in the remaining allele, either. It is more than plausible, therefore, that an epigenetic phenomenon, such as hypermethylation of the promoter region[28] and decreased protein synthesis or increased protein turnover,[26] might be inactivating the remaining allele. Similarly, for the tumor (case 45) with decreased staining but no structural *PTEN* deletion, similar hypotheses may be raised. Other explanations include point mutations in the putative promoter of the remaining allele or normal tissue contamination of the breast samples, thus giving pseudo-hemizygosity in the face of real homozygous deletion. The latter can be discarded because very careful microdissection of the carcinoma components was performed by a pathologist with extensive experience in microdissection. Further, since the pattern of all positive and negative tumors was homogeneous, regional *PTEN* deletions in tumor subclones are very unlikely. Conversely, the observation of rare immunopositive tumors ($n = 4$) which appear to have LOH of flanking markers can be plausibly explained as well: at least in 3 informative tumors, no deletion of the gene proper or no deletion of most of the 3′ end of the gene has occurred. Hence, the monoclonal antibody, which is raised against the C terminus of PTEN, would still immunostain these samples positively. In this situation, therefore, incomplete 5′ deletion of *PTEN* might still be associated with translation of a truncated immunocompetent PTEN protein. In summary, while structural deletion or mutation of *PTEN* can lead to decreased PTEN protein levels, other mechanisms which lead to complete loss of PTEN expression seem to be prominent as well, at least in the breast carcinoma model.

Whether loss of PTEN expression is an early or late event in breast carcinogenesis is still controversial, although preliminary reports suggest that it is a late event.[11] The observation that loss of PTEN expression is correlated with a negative estrogen and progesterone status and that both grade I tumors had strong PTEN expression also strengthen this hypothesis. There is no doubt that these latter clinicopathological observations need to be investigated further. Nonetheless, these data *in toto* argue that despite the observation that germline *PTEN* mutations cause Cowden syndrome,[4] somatic *PTEN* mutation or functional loss of PTEN expression is associated with tumor progression and not tumor initiation, at least in the breast cancer model. It is also clear from our and other data that breast carcinogenesis does not rely uniformly on the involvement of the PTEN pathway, although how PTEN plays a role in various aspects of normal development and in the pathogenesis of breast carcinoma is not straightforward.

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Commentary

Will the real Cowden syndrome please stand up: revised diagnostic criteria

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Cowden syndrome (CS, MIM 158350) is an autosomal dominant disorder with age related penetrance characterised by multiple hamartomas and a high risk of breast, thyroid, and perhaps other cancers. These hamartomas can arise in tissues derived from all three embryonic germ cell layers, in accordance with the prominent expression of the susceptibility gene throughout human embryonic and fetal development.[1] The cardinal features of CS include trichilemmomas, which are hamartomas of the infundibulum of the hair follicle, and mucocutaneous papillomatous papules, which occur in the great majority (>90%) of affected subjects.[2,3] Lesions in the breast or thyroid occur in at least two thirds of CS cases. The incidence of CS before gene identification was estimated to be 1 in a million in a population based Dutch clinical epidemiological study.[2,4] However, after gene identification, this figure was revised to 1 in 200 000,[5] which is almost certainly an underestimate. This is because CS has variable expression and often can have nothing but subtle skin signs, and so this condition is difficult to recognise and remains underdiagnosed. Before 1996, little was known about the molecular aetiology of the inherited hamartoma syndromes, including CS. For purposes of localising the CS gene, the International Cowden Consortium proposed a set of operational diagnostic criteria to ascertain CS families and to assign affected status within families (table 1).[4,6] These criteria have been adopted by the US based National Comprehensive Cancer Network (NCCN) Genetics/High Risk Cancer Surveillance Panel, whose task is to present evidence based or expert consensus practice guidelines.[7]

The susceptibility gene for CS was mapped to 10q22-23 and identified a year later as *PTEN*.[4,8] PTEN is an almost ubiquitously expressed dual specificity phosphatase which acts as a tumour suppressor[9–11] by mediating cell cycle arrest or apoptosis or both, among other as yet unelucidated functions.[12–14] When CS families and cases are ascertained strictly by the Consortium criteria (table 1), the *PTEN* mutation frequency is approximately 80%.[8,15] However, when these criteria are not used, the mutation frequency ranges from 10-50%.[16–18] Bannayan-Riley-Ruvalcaba syndrome (BRR, MIM 153480), an autosomal dominant developmental disorder characterised by macrocephaly, developmental delay, lipomatosis, haemangiomatosis, and speckled penis, is allelic to CS,[19] with a mutation frequency of 50-60%.[20] The highest *PTEN* mutation frequencies (>92%) are consistently obtained in CS-BRR overlap families (Eng and Hampel, 2000, unpublished observations).[20] Recently, a Proteus syndrome-like subject was found to have a germline *PTEN* mutation and a germline mosaic *PTEN* mutation.[21] This Proteus-like patient presented at birth with marked hypertrophy of the right lower extremity in girth and length, pink verrucoid epidermoid naevi in whirls and plaques on the right side of his body, and macrocephaly. The hemihypertrophy progressed such that massive arteriovenous malformations involving the muscles and bones of the entire right lower extremity and pelvis were noted at the age of 6 years. This patient does not meet the diagnostic criteria for Proteus syndrome[22] nor BRR.[23] A de novo germline *PTEN* R335X was found in this case, and non-germline R130X was found in three different non-contiguous affected tissues from the hypertrophied lower extremity.[21] Whether

Table 1 International Cowden Consortium operational criteria for the diagnosis of CS, Ver 1995

Pathognomonic criteria
Mucocutanous lesions
Trichilemmomas, facial
Acral keratoses
Papillomatous lesions
Mucosal lesions
Major criteria
Breast carcinoma
Thyroid carcinoma, especially follicular thyroid carcinoma
Macrocephaly (eg, ≥95th centile)
Lhermitte-Duclos disease (LDD)
Minor criteria
Other thyroid lesions (eg, goitre)
Mental retardation (say, IQ ≤75)
GI hamartomas
Fibrocystic disease of the breast
Lipomas
Fibromas
GU tumours (eg, uterine fibroids) or malformation
Operational diagnosis in a person
(1) Mucocutanous lesions alone if:
(a) there are 6 or more facial papules, of which 3 or more must be trichilemmoma, or
(b) cutaneous facial papules and oral mucosal papillomatosis, or
(c) oral mucosal papillomatosis and acral keratoses, or
(d) palmoplantar keratoses, 6 or more
(2) 2 major criteria but one must include macrocephaly or LDD
(3) 1 major and 3 minor criteria
(4) 4 minor criteria
Operational diagnosis in a family where one person is diagnostic for Cowden syndrome
(1) The pathognomonic criterion/ia
(2) Any one major criterion with or without minor criteria
(3) Two minor criteria

Table 2 International Cowden Consortium operational criteria for the diagnosis of CS, Ver 2000

Pathognomonic criteria
Mucocutanous lesions
Trichilemmomas, facial
Acral keratoses
Papillomatous papules
Mucosal lesions
Major criteria
Breast carcinoma
Thyroid carcinoma (non-medullary), especially follicular thyroid carcinoma
Macrocephaly (megalencephaly) (say, ≥95th centile)
Lhermitte-Duclos disease (LDD)
Endometrial carcinoma
Minor criteria
Other thyroid lesions (eg, adenoma or multinodular goitre)
Mental retardation (say, IQ ≤75)
GI hamartomas
Fibrocystic disease of the breast
Lipomas
Fibromas
GU tumours (eg, **renal cell carcinoma**, uterine fibroids) or malformation
Operational diagnosis in a person
(1) Mucocutanous lesions alone if:
(a) there are 6 or more facial papules, of which 3 or more must be trichilemmoma, or
(b) cutaneous facial papules and oral mucosal papillomatosis, or
(c) oral mucosal papillomatosis and acral keratoses, or
(d) palmoplantar keratoses, 6 or more
(2) 2 major criteria but one must include macrocephaly or LDD
(3) 1 major and 3 minor criteria
(4) 4 minor criteria
Operational diagnosis in a family where one person is diagnostic for Cowden syndrome
(1) The pathognomonic criterion/ia
(2) Any one major criterion with or without minor criteria
(3) Two minor criteria

Operational diagnostic criteria are reviewed and revised on a continuous basis as new clinical and genetic information becomes available.

other Proteus-like cases will have *PTEN* mutations is unknown and is the subject of continuing research. It has been proposed that these syndromes that are defined by germline *PTEN* mutations be collectively termed *PTEN* Hamartoma Tumour Syndrome or PHTS.[20]

In an effort to determine the full clinical spectrum involved in *PTEN* mutation and to confirm the robustness of the Consortium criteria, a study was performed to examine germline *PTEN* mutations in families and subjects ascertained by the minimal presence of breast cancer and any anatomical thyroid disorder in a single person or in a minimum of two first degree relatives in a family but who did not meet the Consortium criteria for the diagnosis of CS.[24] Of 64 CS-like cases ascertained, one was found to have a germline *PTEN* mutation. This family had bilateral breast cancer, follicular thyroid carcinoma, and endometrial adenocarcinoma. There were only four other families with endometrial cancer. These observations suggest that the Consortium criteria are robust and that the small but finite *PTEN* mutation frequency is important in clinical cancer genetic management. Further, it suggests that the presence of endometrial cancer may increase the likelihood of finding germline *PTEN* mutation, even in CS-like families. In another recent study, a nested cohort comprising 103 eligible women with multiple primary cancers within the 32 826 member Nurses' Health Study were examined for the occult presence of germline *PTEN* mutations.[25] Among 103 cases, five (5%) were found to have germline missense mutations, all of which have been shown to cause some loss of function. Of these five, two cases themselves had endometrial cancer. This study, therefore, suggests that occult germline mutations of *PTEN*, and by extrapolation CS, occur with a higher frequency than previously believed. Further, these data confirm the previous observations[24] that endometrial carcinoma might be an important component cancer of CS and, indeed, its presence in a case or family that is reminiscent of CS but does not meet Consortium criteria might actually help increase the prior probability of finding *PTEN* mutation. Taken together, these molecular based observations, together with previous clinical epidemiological studies,[2] were felt sufficient to revise the Consortium criteria for the diagnosis of CS to include endometrial carcinoma (table 2). These revised criteria will most likely be adopted for the next revision of the NCCN document. Although further long term and formal investigation of whether endometrial carcinoma and other tumours are true components of CS, for purposes of research ascertainment and for clinical practice, exponents of CS and the NCCN panel felt that it would be more conservative, and in the interest of the patient, to acknowledge endometrial carcinoma as a component cancer.

Anecdotal evidence suggests that renal cell carcinoma and malignant melanoma may be minor component neoplasias of CS, although the latter association is difficult to prove because melanoma is common in the general population as well. Nonetheless, they should be kept in mind, especially when considering surveillance in PHTS.

Surveillance recommendations are governed by the component tumours of CS, namely, breast carcinoma, non-medullary thyroid carcinoma, adenocarcinoma of the endometrium, renal cell carcinoma, and possibly melanoma. For males and females, annual comprehensive physical examinations paying particular attention to skin changes and the neck (thyroid) region should be instituted at the age of 18 years or five years younger than the youngest diagnosis of a component cancer in the family.[7] For females, annual clinical breast examination and training in breast self examination should begin around the age of 25 years; annual mammography should begin at 30 or five years younger than the earliest age of breast cancer diagnosis in the family.[7] For the next NCCN revised guidelines, the panel would probably also recommend annual surveillance of the endometrium, blind repel (suction) biopsies of the endometrium in the premenopausal years, perhaps beginning at the age of 35 or five years younger than the youngest age of endometrial cancer diagnosis in the family, as well as annual urine analysis for the presence of blood which may be performed together during the annual physical examination. Further, clinicians who look after such families should be mindful to note any other seemingly non-component neoplasia which might be over-represented in a given family.

Who should undergo CS surveillance? Any person known to have a germline *PTEN* mutation (that is, PHTS) should undergo surveillance. Among classical CS and BRR probands, preliminary data suggest that the presence of a *PTEN* mutation is associated with the develop-

ment of breast cancer in any given family.[15 20] Until further data become available, any subject who carries the clinical diagnosis of CS should also undergo surveillance. What is less clear is whether *PTEN* mutation negative BRR should undergo cancer surveillance.

I am deeply grateful to all the patients and families with CS, BRR, and CS-like from around the world who have participated in our studies. I would also like to thank members of my laboratory, numerous collaborators and colleagues, especially Mark Greene and Monica Peacocke, and all the genetic counsellors, especially Heather Hampel and Kathy Schneider, who have contributed in one way or another towards the formulation of these revised criteria. My research activities are funded by the National Institutes of Health, Bethesda, MD, USA, the American Cancer Society, the US Army Breast Cancer Research Program, the Susan G Komen Breast Cancer Research Foundation, and the Mary Kay Ash Charitable Foundation.

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Changes in Endometrial *PTEN* Expression throughout the Human Menstrual Cycle*

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ABSTRACT

Frequent mutation of the *PTEN* tumor suppressor gene in endometrial adenocarcinoma has led to the prediction that its product, a phosphatase that regulates the cell cycle, apoptosis, and possibly cell adhesion, is functionally active within normal endometrial tissues. We examined *PTEN* expression in normal human endometrium during response to changing physiological levels of steroid hormones. *PTEN* ribonucleic acid levels, assessed by RT-PCR, increase severalfold in secretory compared to proliferative endometrium. This suggested that progesterone, a known antineoplastic factor for endometrial adenocarcinoma, increases *PTEN* levels. Immunohistochemistry with an anti-*PTEN* monoclonal antibody displayed a complex pattern of coordinate stromal and epithelial expression. Early in the menstrual cycle under the dominant influence of estrogens, the proliferative endometrium shows ubiquitous cytoplasmic and nuclear *PTEN* expression. After 3–4 days of progesterone exposure, glandular epithelium of early secretory endometrium maintains cytoplasmic *PTEN* protein in an apical distribution offset by expanding *PTEN*-free basal secretory vacuoles. By the midsecretory phase, epithelial *PTEN* is exhausted, but increases dramatically in the cytoplasm of stromal cells undergoing decidual change. We conclude that stromal and epithelial compartments contribute to the hormone-driven changes in endometrial *PTEN* expression and infer that abnormal hormonal conditions may, in turn, disrupt normal patterns of *PTEN* expression in this tissue. (*J Clin Endocrinol Metab* **85:** 2334–2338, 2000)

THE *PTEN* TUMOR suppressor gene is mutated in 34–80% of endometrioid endometrial adenocarcinomas (1–3) and in up to half of premalignant endometrial lesions, atypical endometrial hyperplasias (3–6). Its role in tumor suppression is confirmed by frequent endometrial abnormalities that develop in *PTEN*-deficient mice (7) and the high incidence of breast, thyroid, and endometrial cancers in humans with constitutive mutation of one *PTEN* allele, Cowden's syndrome (8–10). Mutations in the *PTEN* gene have emerged as a primary cause of this most frequent of all gynecological cancers, endometrial adenocarcinoma.

An intriguing feature common to many organs prone to develop somatic *PTEN* mutant tumors is steroid hormone responsiveness. In the case of sporadic endometrial adenocarcinomas, nonphysiological aberrations of sex hormone levels have been repeatedly defined by epidemiological studies as the major risk factor for this disease (11). Is there a relationship between *PTEN* expression and steroid hormone levels that might link the observed high *PTEN* mutational rate and hormonal endometrial risk factors? To date, there is no direct link between steroid hormone response and *PTEN* function. As a primary target organ for sex hormones, the endometrium is an exquisite barometer by which the hormonal environment can be measured. The morphological appearance of endometrium during the latter half of the cycle is sufficiently stereotypical that a trained pathologist can predict the actual menstrual date (±48 h) of a blinded histological specimen. It is thus possible to classify endometrial tissues by histological appearance and infer with a high level of confidence their menstrual age and ambient hormonal conditions.

We have selected normal endometrial tissues from throughout the normal cycle for *PTEN* expression analysis and interpreted our findings in light of the distinctive hormonal profiles that distinguish its phases. Immunohistochemistry permitted further resolution of which cell types contribute to the overall *PTEN* expression within this complex and dynamic tissue.

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Materials and Methods

Tissue samples

Snap-frozen endometrial samples were obtained as discarded materials from hysterectomies of women undergoing surgery for benign, nonendometrial, uterine disease (usually uterine prolapse or fibroids). Endometrial histology was evaluated by review of hematoxylin- and eosin-stained paraffin histological sections obtained at the time of tissue allocation. Endometria from four premenopausal (no exogenous hormone administration, age <50 yr) cycling women included two proliferative and two secretory endometria. An additional hysterectomy specimen from a postmenopausal patient with an atrophic endometrium was included along with myometrium as a control.

Paraffin blocks of histologically normal endometria were retrieved by diagnosis from the pathology files of Brigham and Women's Hospital.

All patients were less than 50 yr old, clinically premenopausal, and without intrinsic endometrial disease or recent history of hormone administration. Histological sections were reviewed by a gynecological pathologist (G.L.M.) for assignment of menstrual date according to a standardized 28-day cycle (12). Day assignments of 40 accessioned normal endometria correspond to sequential hormonal and histological events beginning with the first day of menses as follows: menses, days 1–4 (n = 4); proliferative phase, days 5–15 (n = 8); early secretory endometrium, days 16–18 (n = 7); midsecretory endometrium, days 19–24 (n = 7); and late secretory endometrium, days 25–28 (n = 15).

RT-PCR

RNA was isolated by lysis in guanidine isothiocyanate and selective precipitation with lithium chloride (13). RT of 10 μg total RNA with random hexamers and SuperScript reverse transcriptase (Life Technologies, Inc.>zcomx>-BRL, Gaithersburg, MD) was performed according to the manufacturer's instructions. Identical RNA aliquots underwent parallel manipulation, except for the addition of reverse transcriptase. A constant quantity of resultant complementary DNAs or RNAs without RT was amplified by PCR for 27 PCR cycles at an annealing temperature of 50 C with one of three different *PTEN* primer sets and a control β-actin primer (Research Genetics, Inc., Huntsville, AL; catalogue no. M502) (14). The number of PCR cycles was bracketed between 22–32 to identify a linear range of amplification for the PCR conditions used; 27 cycles was the midlinear range for the primers used. PCR reactions were performed in a 50-μL reaction mix [10 mmol/L Tris (pH 8.4), 50 mmol/L KCl, 20 μg/ml gelatin, 1.5 mmol/L MgCl, oligonucleotide primers 0.1 μmol/L of each, 0.2 mmol/L deoxy (d)-ATP, 0.2 mmol/L dGTP, 0.2 mmol/L dCTP, 0.05 mmol/L TTP, and 50–100 nmol/L [^{32}P]TTP; model PTC-100 thermal cycler, MJ Research, Inc., Cambridge, MA). Oligonucleotide primers for the *PTEN* gene spanned exons 5–7 (PT5-a/b), 6–7 (PT6-a/b), and 8–9 (PT8-a/b). PCR primers are as follows: PT5a, TTTCTATGGGGAAGTAAGGA; PT5b, ACGGCTGAGGGAACTC; PT6a, GTCAGAGGCGCTATGTGTAT; PT6b, GTCTTCCCGTCGTGTG; PT8a, AATGTTTCACTTTTGGGTAA; and PT8b, CGGCTCCTCTACTGTTTTT. PCR products were electrophoresed in 0.4-mm thick polyacrylamide gels under nondenaturing conditions (200–500 V in 8% polyacrylamide gel made in 45 mmol/L Tris-borate and 1 mmol/L ethylenediamine tetraacetate). Gels were dried, and autoradiography was performed using preflashed Kodak XAR film (Eastman Kodak Co., Rochester, NY) at −60 C. Autoradiogram optical density was measured with an EC model 910 optical densitometer (EC Apparatus Corp., St. Petersburg, FL), and the resultant plot was integrated using the GS365W Electrophoresis Data System, version 2.0 (Hoeffer Scientific, San Francisco, CA).

Immunocytochemistry

The monoclonal antibody 6H2.1 (3, 15, 16) raised against the last 100 C-terminal amino acids of *PTEN*, developed and supplied by Jacqueline Lees (Massachusetts Institute of Technology, Cambridge, MA), was used in all immunocytochemical analyses. The specificity of this antibody for *PTEN* has been documented previously (15).

Tissue samples were fixed by immersion in buffered formalin and embedded in paraffin following standard histological practices. Four- to 5-mm sections were cut and mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). Immunostaining was performed essentially as previously described (15). In summary, the sections were deparaffinized and rehydrated. Hydrated tissue underwent antigen retrieval for 20 min at 98 C in 0.01 mol/L sodium citrate buffer, pH 6.4, in a microwave oven. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 30 min. After blocking for 30 min in 0.75% normal serum, the sections were incubated with 6H2.1 (dilution, 1:100) for 1 h at room temperature. Negative control slides received buffer only at this step. The sections were washed in phosphate-buffered saline and then incubated with biotinylated horse antimouse IgG followed by avidin peroxidase using the Vectastain ABC elite kit (Vector Laboratories, Inc., Burlingame, CA). The chromogenic reaction was carried out with 3,3′-diaminobenzidine using copper sulfate amplification, which gives a brown reaction product. After counterstaining with methyl green, the slides were evaluated under a light microscope. The intensity of staining was classified separately for the nucleus/nuclear membrane and the cytoplasm and was graded by two independent observers as strong (+++), moderate (++), weak (+), or absent (−).

Results

PTEN RNA abundance (Table 1) increased by a factor of 5 or more in the transition from proliferative to secretory endometrium, as assessed by three independent PCR assays (Fig. 1, assays PT5-a/b, PT6-a/b, and PT8-a/b).

PTEN immunohistochemistry resolved the tissue-specific (*e.g.* endometrial epithelium, stroma, *etc.*) and subcellular localization of *PTEN* protein in cycling complex endometrial tissues. The distribution of *PTEN* immunohistochemical signal was confined primarily to the endometrium functionalis, the superficial or luminal portion of the endometrial thickness that undergoes dramatic morphological change in response to the changing hormonal conditions that define the menstrual cycle. The deeper endometrial basalis tended to have very faint *PTEN* staining regardless of cycle stage (not shown).

The endometrium functionalis expresses *PTEN* protein in both stromal and glandular epithelial cells, with systematic changes in intensity and subcellular localization during the menstrual cycle (Table 2). Beginning with menstrual endometrium, shed tissue aggregates have nuclear signal in stromal cells, but none in epithelium (Fig. 2A). As the functionalis regenerates during the proliferative phase *PTEN* signal becomes widespread in epithelial and stromal compartments (Fig. 2B). In the early secretory phase, newly formed basal secretory vacuoles exclude *PTEN* protein, which is present only in the apical aspect of glandular epithelial cells (Fig. 2C). At this time, the stromal cells maintain *PTEN* expression in a pattern similar to that of the earlier proliferative phase. In the mid- and late secretory phases, glands are essentially depleted of *PTEN* protein (Fig. 2D). A progesterone-induced change in midsecretory stromal cells, decidualization, corresponds to expansion of the cytoplasmic volume and continues through the later secretory interval

TABLE 1. Increase in *PTEN* RNA in progesterone-exposed endometrium

Assay	Proliferative mean (SD), n = 2	Secretory mean (SD), n = 2	SE/PE ratio	*P* (by *t* test)
PT5-a/b	9,636 (3,797)	53,733 (13,506)	5.6	0.047
PT6-a/b	5,868 (165)	28,023 (4,024)	4.8	0.016
PT8-a/b	2,785 (451)	39,672 (1,179)	14.2	0.001
β-Actin	11,370 (7,196)	14,909 (6,278)	1.3	0.653

Densitometry of RT-PCR autoradiographic signal from Fig. 1 proliferative estrogen-exposed (lanes 3 and 4; n = 2) and secretory progesterone-exposed (lanes 5 and 6; n = 2) endometrium was performed, and averaged results for each tissue type were used to calculate the relative increase in expression within secretory compared to proliferative endometrium (SE/PE ratio). Two-tailed *t* test probability is shown for each primer set. Expression of *PTEN* transcripts as indicated by three PT primer sets increases in secretory relative to proliferative endometrium.

(12). Nuclear signal in decidualized stromal cells at this stage becomes increasingly intense (Fig. 2D), and the cytoplasmic staining becomes somewhat variable relative to that in earlier secretory endometrium.

Discussion

Endometrial expression of *PTEN* is not constant throughout the menstrual cycle, but changes in response to the hormonal environment. Our initial assessment of expression using whole tissue as a RNA source for *PTEN* RT-PCR suggested that the postovulatory secretory phase had increased *PTEN* expression relative to the estrogenic proliferative phase. Immunohistochemical localization of *PTEN* protein within endometrial tissues, however, showed a highly complex distribution in multiple cell types and subcellular locations that cannot be simply summarized by a change in total tissue abundance. The endometrial response to progestins is cell type specific and inverse between epithelial and stromal cells. Estrogen-driven, mitotically active, glandular and stromal cells have a high level of ubiquitous *PTEN* expression in both nuclear and cytoplasmic compartments. With the addition of progesterone, epithelial *PTEN* expression declines to a point where after 3–5 days (cycle days 18–19) this protein is completely extinguished to a level below the threshold of detection. The diminution of epithelial *PTEN* expression begins with a polarized loss of *PTEN* protein from the basal aspect of epithelial cells. At this same time, adjacent stromal cells undergoing cytoplasmic expansion as part of a decidualization process collect abundant nuclear and cytoplasmic *PTEN* protein.

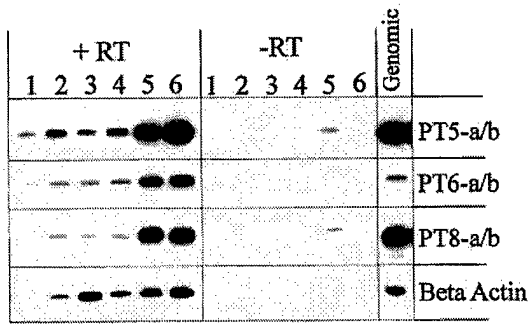

FIG. 1. Endometrial expression of *PTEN* RNA in a changing hormonal environment. *PTEN* expression throughout the normal menstrual cycle was studied by RT-PCR. Equal quantities of normal human endometrial RNA isolated from atrophic (lane 2), estrogen-primed proliferative (2 patients, lanes 3 and 4), and progesterone-exposed secretory (2 patients, lanes 5 and 6) endometria were reverse transcribed (+RT) with random hexamers, and the resultant complementary DNA was used as a PCR template. Myometrium from the postmenopausal patient with atrophic endometrium (lane 2) is included as lane 1. Three different *PTEN* primer sets were used in 27 PCR cycles, spanning exons 5–7 (PT5-a/b), 6–7 (PT6-a/b), and 8–9 (PT8-a/b). All show that *PTEN* RNA levels increased severalfold (see Table I) under progesterone influence (lanes 5 and 6) relative to the estrogenic proliferative phase (lanes 3 and 4) or in the hormonally depleted atrophic state (lane 1). Controls shown include identical RNAs without RT (−RT), genomic DNA, and the constitutively expressed gene β-actin (26). Signal in the +RT lanes can be ascribed to a RNA source, as there is minimal contaminating genomic DNA background (−RT). Each row of data is from a single autoradiogram, with exposure intervals ranging from 4–12 h.

Changes in *PTEN* expression correspond to those endometrial zones that respond to hormonal fluctuation by changes in specialized cellular functions. Areas of endometrium sheltered from cyclical hormone-driven changes have low or absent *PTEN* levels, which remain stable throughout the cycle. This is evident in the endometrial basalis, which does not undergo stromal decidualization or secretory change as seen in more superficial regions (12).

The observation that epithelial *PTEN* expression levels decline in secretory endometrium is unexpected, especially because increasing levels of progesterone are widely known to have antineoplastic effects in this tissue. If *PTEN* had a direct effect on the antitumorigenic properties of progestins, the opposite would be predicted. Two alternate models are worth considering, but will require additional experimentation to evaluate. One is that the *PTEN* effect on endometrial glands is mediated by the adjacent stromal cells. Alternatively, the functional requirement for *PTEN*-mediated tumor suppressor activity might be specific to a highly mitotic estrogenic environment and negated under progestin-dominated conditions that reduce cell division. If this were the case, *PTEN* mutation under unopposed estrogen conditions would result in a high risk of developing carcinoma. This is exactly the combination of circumstances that is known to increase cancer risk: protracted unopposed estrogen exposure (11, 17) and development of a premalignant lesion, many of which we now recognize as having *PTEN* mutations (3). In another study we have shown that endometria stimulated for abnormally long intervals with estrogens begin to display clonal outgrowth of *PTEN*-depleted epithelium, which eventually assumes a physical configuration diagnostic of a precancerous state (3). Correspondingly, pharmacological administration of progestins to patients with endometrial precancers is often effective in causing their ablation, and in primates may increase the expression of tumor suppressor genes such as DMBT1 (18).

TABLE 2. *PTEN* immunohistochemistry in cycling endometrium functionalis

Phase	Day	Epithelium		Stroma		Notes
		Nucleus	Cytoplasm	Nucleus	Cytoplasm	
Menstrual	0–4	−	−	++	−	
Proliferative	5–15	+	+	++	+	
Early secretory	16–18	+/−	+	++	+	Luminal *PTEN*, excluding basal vacuoles
Midsecretory	19–24	−	−	++	++	
Late secretory	25–28	−	−	+++	varies + to +++	Stromal staining of cytoplasm decreases on predecidualization, nuclear strong throughout

PTEN protein signal was evaluated in the superficial endometrium functionalis, and intensity was recorded for each cell type and subcellular compartment on a scale from no expression (−) to intense expression (+++).

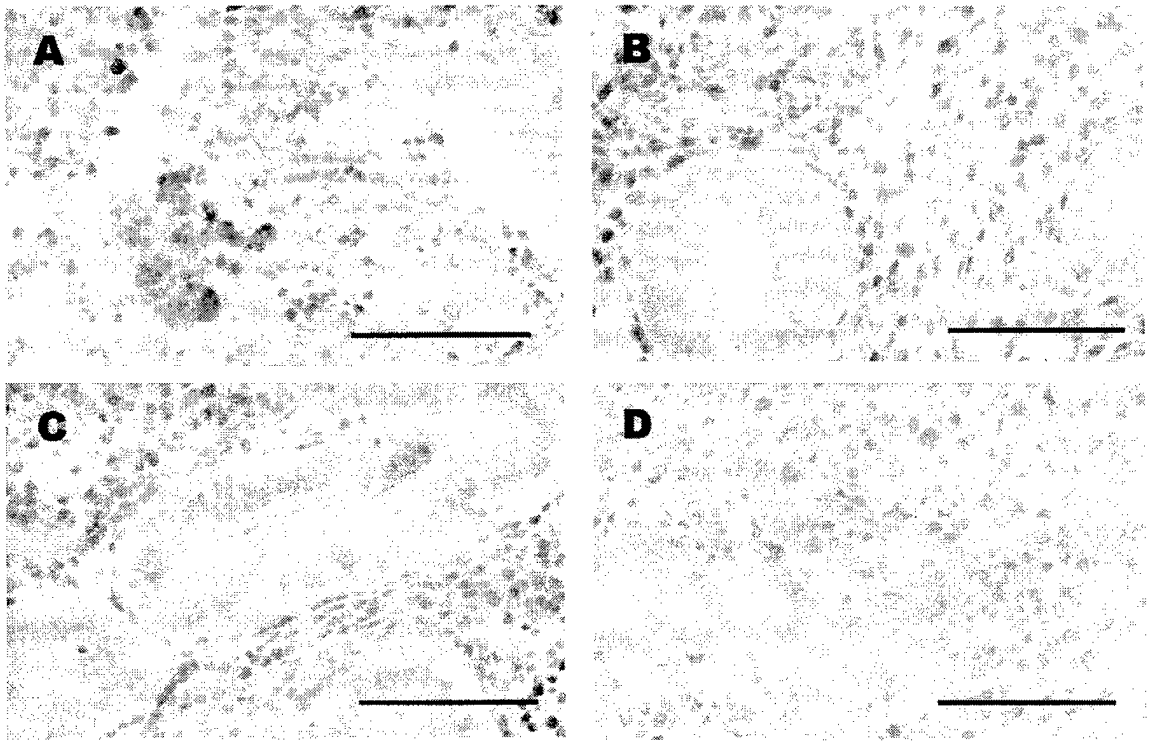

FIG. 2. *PTEN* immunohistochemistry using antibody 6H2.1 displays signal as a *brown* product in sections of menstrual (A), proliferative (B), early secretory (day 17; C), and midsecretory (day 24; D) endometria. *Scale bar*, 100 μm.

A physiological function of *PTEN* exclusive of its postulated role in tumorigenesis is expected. It is essential for complete development, as complete inactivation in knockout mice produces embryonic lethality (7). *PTEN* expression in normal mice is widespread before organogenesis, becoming more restricted thereafter (19), when high levels are seen in skin, breast, thyroid, and brain. These are the very tissues prone to development of neoplasia in adults with acquired or inherited *PTEN* mutations.

Changes in endometrial *PTEN* subcellular localization coincide to shifts in mitotic activity. Mitotically active epithelial and stromal cells have *PTEN* protein in both cytoplasm and nucleus. A relative increase in nuclear localization is seen in nondividing decidualized late stromal cells and apoptotic menstrual stromal cells. To date, *PTEN* has been shown to play some role in cell cycle arrest at the G_1 phase via unknown mediators, apoptosis probably through the PI3K-Akt pathway and cell adhesion via the focal adhesion kinase pathway (20–24). Each of these processes may require *PTEN* to be in specific subcellular localizations. For example, *PTEN* might better regulate cell adhesion and migration through dephosphorylation of focal adhesion kinases in the cytoplasmic compartment (25). If *PTEN* indeed serves to check uncontrolled mitotic division and initiate apoptosis, the fact that these functions are not effective throughout the menstrual cycle requires that *PTEN* expression be coordinated carefully throughout.

In conclusion, *PTEN* expression in normal endometrium is ubiquitous in the estrogenic proliferative phase, but undergoes cell type-specific changes in response to progesterone. Epithelial cells lose *PTEN* protein in the secretory phase, whereas stromal cells increase *PTEN* expression, especially in the cytoplasmic compartment. Epithelial *PTEN* function is probably restricted to the mitotically active glandular epithelium, where its loss by mutation under protracted estrogenic conditions may initiate genesis of a precancerous lesion.

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Altered PTEN Expression as a Diagnostic Marker for the Earliest Endometrial Precancers

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Background: **PTEN tumor suppressor gene mutations are the most frequent genetic lesions in endometrial adenocarcinomas of the endometrioid subtype. Testing the hypothesis that altered PTEN function precedes the appearance of endometrial adenocarcinoma has been difficult, however, partly because of uncertainties in precancer diagnosis.** ***Methods:*** **Two series of endometrial cancer and precancer (endometrial intraepithelial neoplasia, as diagnosed by computerized morphometric analysis) tissue samples were studied, one for PTEN mutations by the use of denaturing gradient gel electrophoresis and another for PTEN protein expression by immunohistochemistry. Endometria altered by high estrogen levels that are unopposed by progestins—conditions known to increase cancer risk—were also studied by immunohistochemistry. Fisher's exact test was used for statistical analysis.** ***Results:*** **The PTEN mutation rate was 83% (25 of 30) in endometrioid endometrial adenocarcinomas and 55% (16 of 29) in precancers, and the difference in number of mutations was statistically significant (two-sided $P = .025$). No normal endometria showed PTEN mutations. Although most precancers and cancers had a mutation in only one PTEN allele, endometrioid endometrial adenocarcinomas showed complete loss of PTEN protein expression in 61% (20 of 33) of cases, and 97% (32 of 33) showed at least some diminution in expression. Cancers and most precancers exhibited contiguous groups of PTEN-negative glands, while endometria altered by unopposed estrogens showed isolated PTEN-negative glands.** ***Conclusions:*** **Loss of PTEN function by mutational or other mechanisms is an early event in endometrial tumorigenesis that may occur in response to known endocrine risk factors and offers an informative immunohistochemical biomarker for premalignant disease. Individual PTEN-negative glands in estrogen-exposed endometria are the earliest recognizable stage of endometrial carcinogenesis. Proliferation into dense clusters that form discrete premalignant lesions follows. [J Natl Cancer Inst 2000;92:924–31]**

Somatic mutation or deletion of the PTEN tumor suppressor gene has been reported in approximately 40% *(1,2)* and 40%–76% *(3,4)*, respectively, of endometrial adenocarcinomas. Further evidence for PTEN function within the female reproductive tract is evident in pten knockout (null mutant) mice that develop complex proliferative endometrial lesions *(5)*. In humans, familial inheritance of mutant PTEN alleles in Cowden syndrome causes multiorgan development of benign hamartomatous and malignant epithelial tumors *(6–8)*, including an elevated incidence of endometrial adenocarcinoma (Eng C, Peacocke M: unpublished observations).

Patients with endometrioid endometrial adenocarcinoma *(1,2)* account for 80% of endometrial cancer patients in the United States *(9,10)*. Among all histologic subtypes of endometrial adenocarcinomas, the endometrioid subtype appears to have the highest rate of somatic PTEN mutations *(1,2)*. Routine histopathology readily discriminates endometrioid endometrial adenocarcinomas from nonendometrioid tumors, such as the papillary serous and clear-cell adenocarcinomas that also occur in the endometrium. Risk for endometrioid endometrial adenocarcinomas is increased in patients with high estrogen levels that are unopposed by progestins *(11)* and in patients with a physically distinctive precancerous lesion *(12)*. Interaction between genetic and hormonal events during the premalignant phases of endometrial tumorigenesis has been hypothesized, yet it has never been precisely elucidated.

The inaccessibility of premalignant tissues, the controversy concerning their interpretation, and the paucity of high-yield candidate genes are long-standing—but now fast-disappearing—barriers to productive exploration of the biology of endometrial precancers. Polymerase chain reaction (PCR)-based methods, including detailed mutational *(13)*, clonal *(14)*, and even lineage reconstruction *(15)* analyses, have improved the analytic repertoire suited to physically small precancers. Accurate diagnosis of the precancers themselves, typically termed "hyperplasias" in the widely used World Health Organization nomenclature *(16)*, has been difficult to standardize *(17)*. Even when criteria are agreed upon, reproducibility *(18)* is suboptimal. Previous reports of PTEN mutations in putative endometrial precancers *(19–21)* have used subjective diagnostic criteria. Objective computerized morphometry *(12,22,23)*, which uses image analysis algorithms that have excellent ability to predict concurrent *(23)* or future *(12)* carcinoma, has been shown to improve the reproducibility of histopathologic diagnoses. We have previously validated computerized morphometric analysis as an accurate means of precancer identification by showing that most of the lesions that it classifies as precancers are, in fact, monoclonal neoplasms *(24)*, albeit benign ones prone to malignant transformation.

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See "Note" following "References."

We have performed PTEN mutation analysis and protein expression studies in a spectrum of precisely classified endometrial tissues to test our hypothesis that changes in PTEN structure and function are among the earliest events in the pathway to endometrioid endometrial cancer. A series of unopposed estrogen-exposed endometria was included to determine if altered PTEN function might precede the appearance of endometrial intraepithelial neoplasia (EIN), which we define as a precancer diagnosed by morphometry.

Materials and Methods

Tissue Samples

Two separate series of paraffin-embedded endometrial tissue samples were selected from the pathology files of Brigham and Women's Hospital (Boston, MA) by report review for diagnoses of endometrial adenocarcinoma and/or anovulatory–hyperplastic endometrium after approval was received from the Human Studies Committee at that institution. The first series of samples, hysterectomy specimens from 30 patients with endometrioid endometrial adenocarcinoma and premalignant lesions ("precancers") that were diagnosed objectively by computerized morphometric analysis, was used for PTEN mutational analysis. A single region representative of each tissue diagnosis was selected in each sample. Ten of these hysterectomy specimens also contained histologically normal endometrium suitable for analysis, and all 30 contained normal myometrial tissue for use as a DNA control. Endometrial polyps were excluded from the analysis. The second series of samples, endometrial tissues from 54 patients (34 hysterectomy specimens and 20 curetting/biopsy specimens), was used for PTEN immunohistochemistry. The samples used for immunohistochemistry were all less than 1 year old, whereas most of the first series of hysterectomy specimens (used for mutational analysis) were from surgeries performed more than 2 years earlier.

Histologic Classification by Use of Computerized Morphometric Analysis

Diagnostic classification was accomplished by a combination of review by a pathologist (G. L. Mutter) and computerized morphometry. First, carcinomas were distinguished from premalignant lesions by the presence of at least one of three diagnostic features: 1) myometrial invasion, 2) solid areas of neoplastic epithelium, or 3) extensively meandering, interconnected glandular structures. Endometrial tissues that were judged not to constitute carcinomas were circumscribed with ink on the glass slide. Computerized morphometric analysis of corresponding delineated regions on hematoxylin–eosin-stained sections was performed (by J. P. A. Baak) by use of the QProdit 6.1 system (Leica, Cambridge, U.K.) as described previously *(12,23,25)*. For each lesion, the D score was calculated from the volume percentage stroma (VPS), standard deviation of shortest nuclear axis (SDSNA), and gland outer surface density (OUTSD) [$D = 0.6229 + (0.0439 \times VPS) - (3.9934 \times \ln[SDSNA]) - (0.1592 \times OUTSD)$ *(12,23)*] and was then classified as precancerous (EIN) ($D<0$), indeterminate ($0 \leq D \leq 1$), or benign ($D>1$) based on the previously developed *(12,23)* outcome-predictive formula. Endometrial areas scored as benign were subclassified by pathologist (G. L. Mutter) review. Atrophic, cycling, or reactive endometrium was identified and grouped as "normal." Unopposed estrogen-exposed endometria were diagnosed by the appearance of occasional glandular cysts in a disordered proliferative field without sufficient glandular crowding or atypia to qualify as a precancer. The source of unopposed estrogen was either endogenous (anovulatory cycles) or exogenous (pharmacologic estrogens).

DNA Isolation and Amplification

Genomic DNA from endometrial tissues (normal, precancer, or cancer) obtained at hysterectomy was isolated by selective UV irradiation *(14)* of areas of the paraffin sections that were typically 3 mm in diameter and contained dozens of individual glands. PTEN-coding sequences were amplified by PCR by use of target-specific oligodeoxynucleotide primers. Intron-based PCR primers were used to minimize coamplification of the processed (intronless) PTEN pseudogene on chromosome 9 *(26)*. In the following list of the primers that we used, each like-numbered pair comprises the forward ("FGC") and reverse ("RGC") primers for the correspondingly numbered PTEN exon: 1FGC (5′-CGT CTG CCA TCT CTC TCC TCC T-3′), 1RGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT CCG TCT ACT CCC ACG TTC T-3′), 2FGC (5′-CGT CCC GCG TTT GAT TGC TGC ATA TTT CAG-3′), 2RGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTC TAA ATG AAA ACA CAA CAT G-3′), 3FGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTA AAT GGT ATT TGA GAT TAG-3′), 3RGC (5′-GCG CGA AGA TAT TTG CAA GCA TAC A-3′), 4FGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAA ATA ATA AAC ATT ATA AAG ATT CAG GCA ATG-3′), 4RGC (5′-GAC AGT AAG ATA CAG TCT ATC-3′), 5.1FGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTT TTT TCT TAT TCT GAG GTT ATC-3′), 5.1RGC (5′-TCA TTA CAC CAG TTC GTC C-3′), 5.2FGC (5′-TCA TGT TGC AGC AAT TCA C-3′), 5.2RGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGAA GAG GAA AGG AAA AAC ATC-3′), 6FGC (5′-GCG CGT TTC AAT TTG GCT TCT CTT T-3′), 6RGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT AAG AAA ACT GTT CCA ATA C-3′), 7FGC (5′-CGT CCC GCA ATA CTG GTA TGT ATT TAA C-3′), 7RGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GGA TAT TTC TCC CAA TGA AAG-3′), 8FGC (5′-CGG TTT CAC TTT TGG GTA AAT A-3′), 8RGC (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC CCC CAC AAA ATG TTT AAT-3′), 9FGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTC ACT AAA TAG TTT AAG ATG-3′), and 9RGC (5′-TTC ATT CTC TGG ATC AGA GT-3′). Since each sense primer had a 30- to 45-base-pair GC clamp at its 5′ end (e.g., primer 1RGC), each amplicon (PCR product) included a domain with a lower melting temperature (the sequence to be analyzed for mutations) and a domain with a higher melting temperature (the GC clamp).

Detection and Sequence Analysis of PTEN Mutations

Denaturing gradient gel electrophoresis (DGGE) separates amplicons on the basis of melting temperature, which varies with nucleotide composition. We used a 10% polyacrylamide gel containing 5% glycerol and a linear 15%–50% urea–formamide gradient, which simulates a temperature gradient, and subjected our samples to electrophoresis at 100 V for 16 hours at 60 °C *(27)*. Under these conditions, a double-stranded PCR product moves through the gel until it reaches the level corresponding to the melting temperature of its lower melting domain, whereupon that domain melts instantly. Since the high-melting GC clamp holds the amplicon together, migration ceases. PCR products of mutant DNAs exhibit altered migration during DGGE and usually appear as doublets of mutant PTEN products admixed with wild-type DNA that was contributed by contaminating normal tissues or the companion allele.

PCR products were visualized by UV transillumination of ethidium bromide-stained gels. DNA was isolated from bands identified as aberrant and was further amplified, and a nested sequencing primer was used to generate fluorescence-labeled sequencing products that were analyzed on a semiautomated DNA sequencer (ABI377; Perkin-Elmer Corp., Norwalk, CT) as described previously *(6)*. DGGE gels and sequencing chromatograms were independently read by J. B. Kum, C. Eng, and J. T. Fitzgerald or G. L. Mutter.

Analysis of Loss of Heterozygosity

DNA from carcinoma and adjacent normal myometrium was amplified in the presence of [α-^{32}P]thymidine 5′-triphosphate with primers that define D10S541 and D10S215 (MapPairs; Research Genetics, Huntsville, AL), which are polymorphic microsatellite loci at the 3′ and 5′ ends, respectively, of PTEN. PCR products of these polymorphic microsatellites were separated on nondenaturing polyacrylamide gels *(28,29)*. The intensities of bands representing PCR products of tumor alleles were visually compared with those on a reference set of calibrated autoradiographs *(30)* of normal myometrium and scored as positive for loss of heterozygosity (LOH) when there was at least a 50% reduction in the intensity of the band corresponding to one allele.

Immunohistochemistry

Monoclonal antibody 6H2.1, raised against a 100-amino acid oligopeptide identical to the C-terminal end of human PTEN protein *(31)*, was used in all of the immunocytochemical analyses. Specificity has been demonstrated previously by western blot analysis of wild-type and PTEN-null cell lines *(31)*. Furthermore, when the PTEN-specific antibody was incubated with competing synthetic PTEN peptide (the native antigen) and used to immunostain paraffin-embedded sections of known PTEN-expressing tissues, no immunostaining was observed *(31)*.

Since PTEN immunohistochemistry by use of the 6H2.1 antibody requires freshly cut paraffin sections from recently embedded (within 6–12 months) tissues to maximize the signal, we used our second

series of endometrial tissue samples, which met this requirement, for immunohistochemistry. Formalin-fixed tissue samples were embedded in paraffin by standard histologic procedures. Immunostaining was performed by use of a microwave antigen-retrieval protocol as described previously *(31)*. Sections were incubated with monoclonal antibody 6H2.1 (dilution 1 : 100 in phosphate buffer) for 1 hour at room temperature, washed, and incubated with a secondary biotinylated horse anti-mouse immunoglobulin G (Vecstatin ABC kit; Vector Laboratories, Inc., Burlingame, CA). PTEN expression, as reflected by immunostaining, was detected by sequential addition of avidin peroxidase (Vector Laboratories, Inc.) and 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO), which gives a brown reaction product. The intensity of the epithelial staining was scored (by G. L. Mutter and J. T. Fitzgerald) in methyl green-counterstained slides from 0 (absent) to 3 (intense). Endometrial stroma and/or normal endometrial epithelium provided an internal positive control, and negative controls without addition of primary antibody showed low background staining in all cases.

Statistical Analysis

Fisher's exact tests were performed by use of SYSTAT v. 9.0 (Statistical Package for Social Sciences, Chicago, IL). All P values are two-sided.

Results

To determine the earliest stage of endometrial neoplasia in which PTEN mutation occurs, we examined 30 hysterectomy specimens containing endometrioid endometrial adenocarcinomas as well as coexisting computerized morphometry-diagnosed benign or premalignant endometrial tissue for the presence of mutations. Somatic (occurring in tumor only) PTEN mutations were found in 25 (83%) of 30 endometrial cancers and in 16 (55%) of 29 precancers (Table 1). Fisher's exact test of diagnosis (endometrioid cancer versus precancer) by PTEN mutation (present versus absent) showed that cancers had a statistically significant (P = .025) increased number of PTEN mutations compared with their precursors.

None of the 10 samples of normal endometria that we examined showed mutations in PTEN. It is interesting that, among both cancers and precancers, the majority (73% [22 of 30] and 52% [15 of 29], respectively) harbored a mutation in only one exon, but intragenic mutations affecting at least two exons were also observed (Table 1). Fig. 1 shows the number of mutant PTEN exons in 39 nonmalignant tissues that were clearly segregated, by computerized morphometric analysis, into the precancerous (EIN) (D score <0) or benign (D score >1) groups and shows how the D scores of mutations in the two groups are distributed.

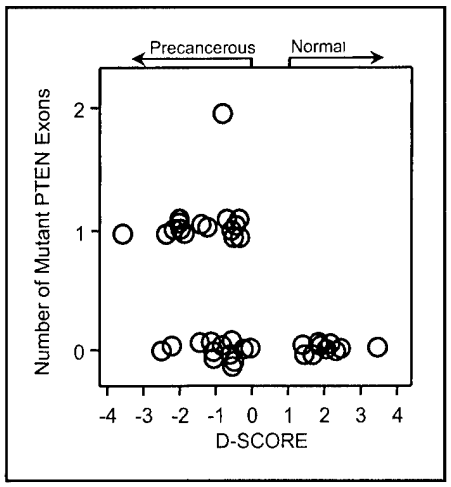

Fig. 1. PTEN mutation and lesion classification by computerized morphometry. Thirty-nine nonmalignant tissues were classified as precancerous or benign on the basis of computerized morphometric D scores, as defined in the text *(12,23)*. Each **circle** shows the D score and the number of mutant PTEN exons detected in a single tissue sample. Symbols are slightly randomly jittered to improve visibility of overlapping symbols.

We found three mutations deep within

Table 1. Number of PTEN mutations and immunohistochemical assessment of PTEN protein expression in endometrial tissue samples

	PTEN mutations*			PTEN protein expression†				
		No. (%) of PTEN mutations			Immunohistochemical assessment of staining, No. (%)			
Endometrial tissue diagnosis	No. of samples	Any mutation	Mutations in ≥2 exons	No. of samples	Absent	Mild	Moderate	Intense
Endometrioid cancer	30	25 (83)	3 (10)	33	20 (61)	4 (12)	8 (24)	1 (3)
Precancer (EIN)‡	29	16 (55)	1 (3)	12	9 (75)	1 (8)	2 (17)	0
Indeterminate§	ND	ND	ND	9	5 (56)	1 (11)	3 (33)	0
Unopposed estrogen effect‖	ND	ND	ND	7	2 (29)	0	2 (29)	3 (43)
Normal¶	10	0	0	20#	1 (5)	9 (45)	9 (45)	1 (5)
Nonendometrioid cancer	ND	ND	ND	8**	2 (25)	2 (25)	2 (25)	2 (25)

*Genomic DNA from 30 hysterectomy specimens containing endometrioid endometrial adenocarcinoma and premalignant lesions ("precancers") was amplified by the polymerase chain reaction by use of primers for nine PTEN exon mutations, and mutations detected by denaturing gradient gel electrophoresis were confirmed by direct sequencing. One precancer area failed to amplify, and PTEN mutations were also analyzed in an additional 10 regions of histologically normal endometrium in these same hysterectomy specimens. ND = no data.

†Formalin-fixed endometrial tissues from 54 patients (34 hysterectomy specimens and 20 curetting/biopsy specimens) were embedded in paraffin and imunostained with antibody 6H2.1, which detects PTEN protein, and the epithelial/glandular cells were scored.

‡Diagnosed as precancerous (D [defined in text] <0) by computerized morphometry. All samples were independently confirmed as endometrial intraepithelial neoplasia (EIN) by the pathologist (G. L. Mutter).

§Diagnosed as indeterminate ($0 \leq D \leq 1$) by computerized morphometry. Diagnosed as EIN (six of nine), unopposed estrogen (one of nine), secretory endometrium (one of nine), or unknown (one of nine) by the pathologist (G. L. Mutter).

‖Diagnosed as benign (D>1) by computerized morphometry, with stigmata of unopposed estrogen.

¶Diagnosed as benign by computerized morphometry; included atrophic, inactive, or cycling endometrium.

#Unstained glands were always admixed with stained glands. One severely atrophic endometrium contained no discernible PTEN protein.

**Two undifferentiated carcinomas, four papillary serous carcinomas, and two malignant mixed Müllerian tumors.

introns, but they are not included in the data shown in Table 1 or in Fig. 1 because they are unlikely to have any functional impact. A detailed listing of mutations found is available at www.jnci.oupjournals.org/content/vol92/issue11/.

PCR-based analysis to determine LOH of markers within or flanking PTEN was performed on the series of 30 endometrial carcinoma samples shown in Table 1. Overall, the LOH frequency was 23% (seven of 30) (data not shown), and all samples with LOH had PTEN mutations in the remaining allele, indicating inactivation of both PTEN alleles. Attempts to perform LOH analysis on precancers were confounded by the presence of contaminating normal stromal tissue.

The number of genetically altered PTEN alleles within individual endometrial adenocarcinomas can be estimated by combining deletion (LOH) and mutation (DGGE, Table 1) data. Ten (33%) of 30 endometrioid carcinomas had homozygous PTEN inactivation (seven with LOH of one allele and mutation of the second allele and three with mutations in two or more PTEN exons), and another 50% (15 of 30) had hemizygous PTEN genomic lesions (DGGE-detected mutation in one allele only, without LOH of second allele).

Probable biallelic inactivation of PTEN is reflected in lack of PTEN protein expression, which can be assessed by immunohistochemistry. Fig. 2 shows immunohistochemical detection of PTEN protein (brown precipitate) by antibody 6H2.1 in areas of endometrial adenocarcinoma, endometrial precancer, and benign endometrium. Although all of the tissue samples shown in Fig. 2 are from one patient, it illustrates the salient PTEN immunohistochemical findings that are typical of malignant, premalignant, and estrogen-driven endometria from the 81 (excluding nonendometrioid cancers) endometrial tissue samples that we have examined. The carcinoma in Fig. 2, A, is devoid of PTEN staining, but adjacent endometrial stromal cells and vascular endothelium contain cytoplasmic and nuclear PTEN protein. A zone of precancerous glands devoid of PTEN protein (Fig. 2, B; upper left) contrasts with abundant stromal staining and an adjacent region of normal endometrial glands (Fig. 2, B; lower right) that show both nuclear and cytoplasmic PTEN staining. The high-magnification views (Fig. 2, C and E) of the upper-right corner of Fig. 2, B, show the interface between PTEN-negative (precancerous) and PTEN-positive (benign) glands, including one transition within an individual gland (Fig. 2, E). Companion hematoxylin–eosin-stained sections (Fig. 2, D and F) are the equivalent of those immunostained with anti-PTEN antibody and show the histologic structure of the tissues.

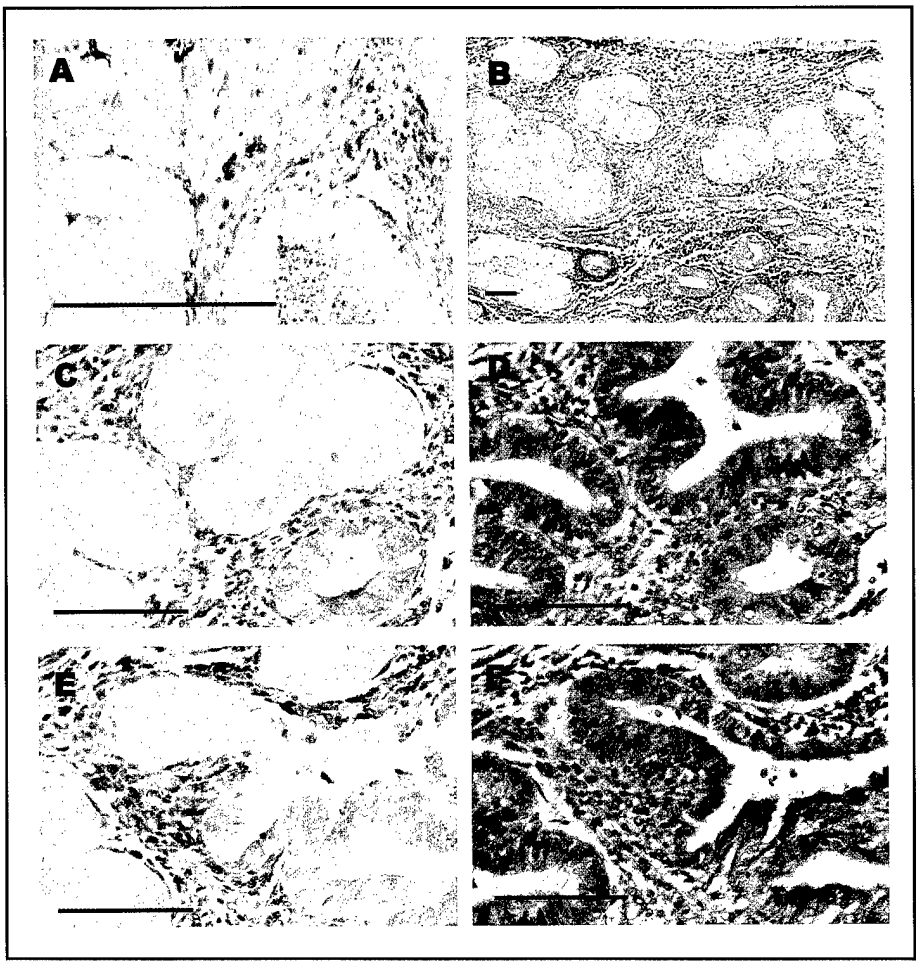

Fig. 2. PTEN protein in endometrial cancer and precancerous endometrial intraepithelial neoplasia. Immunohistochemical staining (**brown**) of PTEN protein with antibody 6H2.1 in **A**) endometrial adenocarcinoma; **B**) a geographic zone of precancerous glands (**upper half and left**) contrasting with an adjacent region of normal endometrial glands (**lower right**); **C**) higher power view of upper right region of panel B; and **E**) transition from PTEN-expressing to nonexpressing epithelium within an individual gland. Companion hematoxylin–eosin-stained serial sections of panels C and E are shown in panels **D** and **F,** respectively. **Scale bar** is 100 μm.

Whereas most unopposed estrogen-exposed endometria showed ubiquitous epithelial PTEN protein expression, 29% (two of seven) had a background of PTEN protein-positive glands punctuated by scattered negative glands. Fig. 3 shows endometria with heterogeneous PTEN protein expression. It demonstrates scattered PTEN-negative glands that are interposed among PTEN-expressing glands to present an interrupted pattern that is different from the geographic distribution within the (monoclonal) readily diagnosed precancers shown in Fig. 2. This intermittent pattern was seen at a variety of gland densities, ranging from the closely packed architecture characteristic of precancers defined by computerized morphometry (Fig. 3, A–C) to the low densities of a disordered proliferative endometrium (unopposed estrogen effect) (Figs. 3, D–F). The cytology of PTEN-nonexpressing glands may be similar to (Fig. 3, B and C) or different from (Fig. 3, E, versus Fig. 3, F) that of surrounding expressing glands. Panels G and H of Fig. 3 show a persistent estrogen-exposed endometrium characterized by cysts, which retains epithelial and stromal PTEN expression. Most areas of tubal change in estrogen-driven, disordered proliferative endometrium continue to express PTEN protein. Companion hematoxylin–eosin-stained sections (Fig. 3, C and F) are the equivalent of those in Fig. 2.

Six diagnostic classes of endometrial

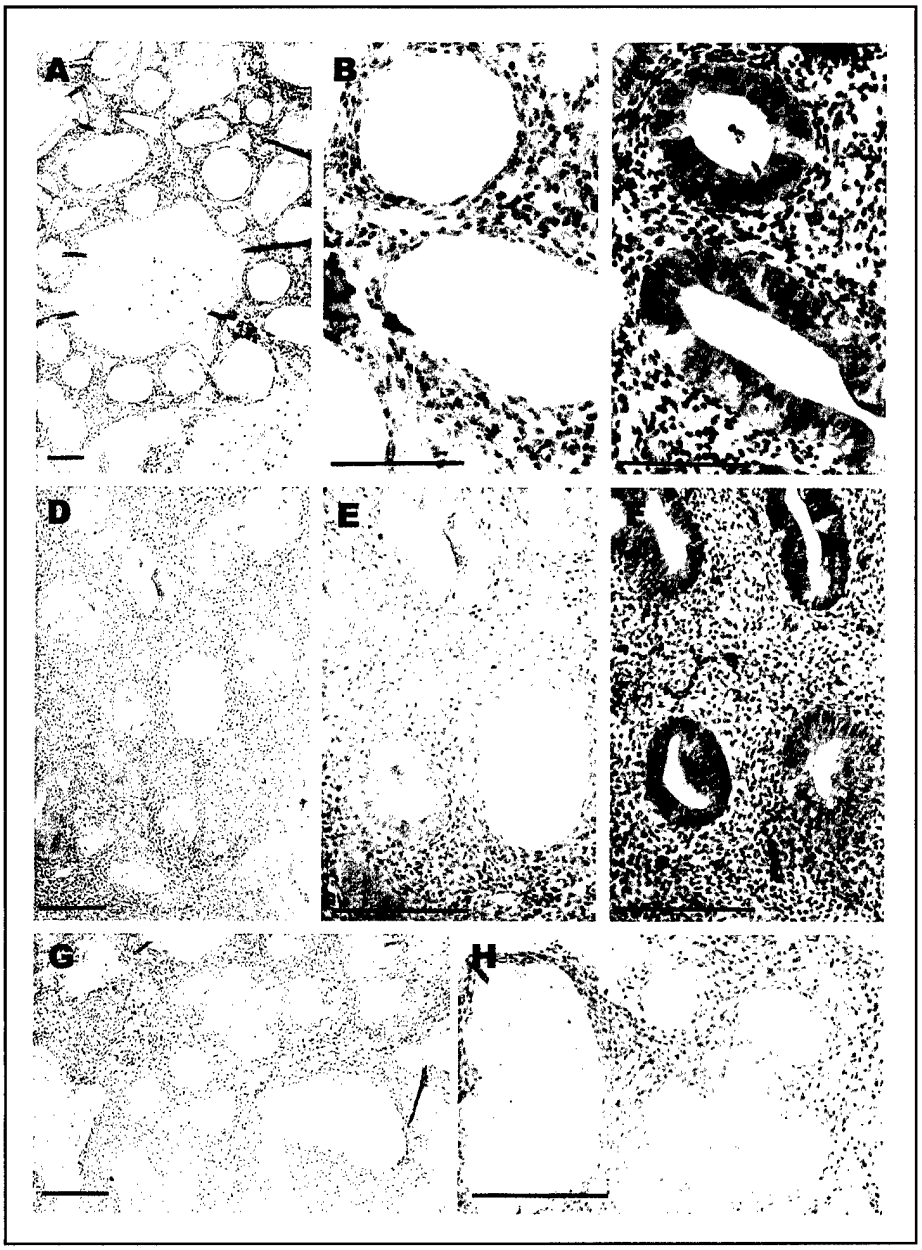

Fig. 3. PTEN protein heterogeneity in precancers and endometria with unopposed estrogen effect. Immunohistochemical staining (**brown**) of PTEN protein with antibody 6H2.1 of **A**) closely packed glands characteristic of precancers defined by computerized morphometry (isolated glands are PTEN negative); **B**) higher power view of panel A; **D**) disordered proliferative endometrium (unopposed estrogen effect) with scattered PTEN-negative glands; **E**) higher power view of panel D; **G**) persistent estrogen-exposed endometrium characterized by cysts, retaining epithelial and stromal PTEN expression throughout; and **H**) higher power view of panel G. Companion hematoxylin–eosin-stained serial sections of panels B and E are shown in panels **C** and **F**, respectively. **Scale bar** is 100 μm.

tissue samples were assessed by immunohistochemistry for PTEN protein expression (Table 1). Of all the endometrioid endometrial cancers tested, 97% (32 of 33) showed either complete absence of or reduced PTEN protein expression. PTEN protein expression was absent from endometrioid endometrial adenocarcinomas more frequently (20 [61%] of 33 samples) than it was absent from nonendometrioid carcinomas (two [25%] of eight samples). Because of the small sample size, however, the difference in PTEN expression (absent versus present at any intensity) as a function of tumor type (endometrioid versus nonendometrioid) was not statistically significant ($P = .115$).

Of nine computerized morphometry-defined indeterminate endometrial samples, five (56%) showed no PTEN protein expression. Of 20 morphometrically diagnosed normal tissues, only one had no PTEN expression. This was an atrophic endometrium.

Both adjacent endometrial stroma and endothelial cells of blood vessels in immediate proximity to the tumor were moderately PTEN protein positive. (Fig. 2, A, is a typical example.) Precancerous (EIN) lesions had no discernible PTEN protein expression in 75% (nine of 12) of the examples, most commonly in closely packed expanses of PTEN-negative glands offset by dispersed benign glands having a different cytology. (Panels B and C in Fig. 2 are examples.) A less frequent pattern of heterogeneous PTEN staining was seen in some "transitional" benign–precancer examples without cytologic changes (Fig. 3, A–C). No statistically significant difference in PTEN protein expression (absent versus present at any intensity) as a function of diagnosis (cancer versus EIN) was observed ($P = .491$), although the small sample size (33 cancers and 12 EIN lesions) limits the power of this comparison.

Discussion

Based on the current results, it is clear that loss of PTEN function begins in the earliest stages of endometrial tumorigenesis, under conditions of unopposed estrogen exposure that have long been known *(11,32,33)* to increase cancer risk. We have found some endometria with protracted estrogen stimulation unopposed by progestins in which individual glands have already ceased production of PTEN protein. Contiguous expanses of tightly packed glands may also be PTEN negative; these are precancerous lesions that have been shown previously to be monoclonal *(24)*. Our results showed that the PTEN mutation rate in precancers diagnosed by computerized morphometry, which predict a high likelihood of coexisting or future endometrial carcinoma *(12,23)*, is 55%; that the PTEN mutation rate in endometrioid cancers is 83%; and that the difference is statistically significant. Thus, PTEN inactivation occurs during the initiation of precancers from a normal background state, and additional PTEN damage accumulates in the transition from premalignant to malignant disease. Thus, immunohistochemically detected loss of PTEN expression is an informative biomarker for endometrial neoplasia, including precancerous lesions.

Loss of PTEN protein in nests of crowded endometrial glands follows the predictions of monoclonal growth *(14,34)*, namely, that all cells in the lesion share the same PTEN status. Precancer

diagnosis by use of computerized morphometric analysis requires histologic sections with crowded groups of endometrial glands over a field of several square millimeters. It is these clusters of crowded glands that correspond to premalignant lesions usually diagnosed by pathologists as atypical endometrial hyperplasias and which we have designated as EIN by use of morphometry. In practice, diagnosis of endometrial precancers by use of hyperplasia *(16)* terminology is only modestly reproducible *(17,18,35),* especially for nonatypical hyperplasias, a category containing monoclonal putative precancers and polyclonal benign tissues. Computerized morphometry, however, appropriately classifies "nonatypical" hyperplasias into high- and low-risk subgroups *(24).* Fig. 1 shows that mutations in the computerized morphometry-defined high-risk group (D<0, precancerous) are evenly distributed across a rather broad range of D scores and are not clustered at some extreme negative D score distant from the threshold of 0. This validates the idea that all endometrial specimens with a D score below 0 have a high likelihood of having a PTEN mutation.

More troublesome to diagnose are lesions with histologic features intermediate between benign and precancerous, as represented by the "indeterminate" category in Table 1. It is this group of patients in whom PTEN immunohistochemistry may elucidate clonal loss of PTEN expression among a strongly PTEN-positive background of convoluted and "busy" glands. Immunohistochemical identification of individual isolated PTEN-negative endometrial glands in endometria with unopposed estrogen exposure pushes the limits of detection of precancers to an earlier stage of development than was previously possible. The natural history of individual PTEN-negative glands has not yet been determined experimentally, but a rich epidemiologic literature *(32,33)* showing a 3.1- to 7.3-fold increased risk of endometrial adenocarcinoma in women exposed to unopposed estrogens is consistent with the notion that, in some women, these single glands may progressively expand into histologically recognizable premalignant, and ultimately malignant, processes.

Suppression of PTEN expression in a mitotically active estrogenic environment (unopposed by progestins) may compromise growth control more than loss of PTEN protein in mitotically quiescent cells. Highly mitotic cells, such as normal estrogen-stimulated proliferative endometrial glands, contain abundant PTEN protein. Progesterone, which is known to prevent many of the tumorigenic effects of estrogens, diminishes *in vivo* endometrial epithelial PTEN protein expression over a period of 4–5 days, to a point where it is no longer detected in the glands of mid-secretory endometrium (data not shown). If these fluctuations in PTEN protein are indeed driven by changing physiologic requirements for the protein, then it is reasonable to predict that the reduced ability to make PTEN protein has a greater effect under estrogenic than under progestenic conditions.

PTEN inactivation (loss of protein) in endometrioid adenocarcinomas and in several other tumor types cannot be explained solely on the basis of observed mutations. This observation suggests that expression of PTEN is repressed at the transcriptional and translational levels by other mechanisms. Fewer than 30% of hematologic malignancies have a structural PTEN alteration, but 70% are PTEN negative as judged by western blot analysis *(36).* Forty percent of breast cancers are PTEN genetic hemizygotes, and fewer than 5% of cases have biallelic PTEN genomic lesions, yet 15% are devoid of protein that is detectable by immunohistochemistry *(31).* In this study, inactivation of both PTEN alleles, as a result of either a mutation or a deletion (LOH), was observed in only 33% of endometrial cancers, but 61% of those cancers did not express PTEN protein. More frequent is a hemizygous PTEN genotype in 50% of endometrial cancer cases. Candidate mechanisms for inactivation of the second allele include (undetected) mutation in regulatory regions, epigenetic modification of flanking DNA sequences (e.g., by methylation), or decreased translation. Increased protein degradation in a hemizygous state could also give negative PTEN immunohistochemistry results that would be indistinguishable from biallelic inactivation.

The PTEN mutation rate of 83% that we observed in our series of endometrial adenocarcinomas is about double that of most previous reports *(1,2,19,20),* probably because of the combined effects of our mutation-detection and sample-selection methods. The DGGE-screening method is very sensitive in PTEN mutation detection compared with the single-strand conformational polymorphism analysis, and its specificity has been confirmed by direct sequencing of DGGE-identified mutations *(27).* DGGE can detect variants, even at mutant-to-normal allele ratios of 1 : 100, while sequencing requires this ratio to be at least 35 : 100 [reviewed in *(37)*].

The tissue samples that we used for mutational analysis were selected for the presence of both malignant and premalignant endometrial tissues at the time of hysterectomy, thereby enriching the study population for those tumors that develop through a hierarchy of progressive events. Endometrioid adenocarcinoma and its precursors have severalfold higher PTEN mutation rates than those malignant tumors (nonendometrioid, including papillary serous type) that arise abruptly without displaying an intermediate premalignant phase. It is unlikely that the balance of microsatellite-stable and microsatellite-unstable tumors can explain the high PTEN mutation rate that we observed, since both had similar PTEN mutation rates in our series.

The mechanism of diminished PTEN protein expression was indirectly addressed in our study, in which, for technical reasons, genomic and protein expression PTEN analyses were carried out on independent tissue series. Our ability to relate changes in PTEN expression to causal genomic events is thus inferential and limited by our sample size. Simultaneous scoring of PTEN mutation and deletion against expression in individual tissues would determine whether these mechanisms alone can explain the majority of lost PTEN expression. One advantage of using this particular series of tissue samples for PTEN mutational analysis is that it was subjected previously to a number of specialized analyses. Of the 30 cancers analyzed, 10 were microsatellite unstable and 20 were microsatellite stable *(14,15,24),* with PTEN mutation rates of 90% (nine of 10) and 80% (16 of 20), respectively. In 29 precancers, 57% (12 of 21) of microsatellite-stable and 50% (four of eight) of microsatellite-unstable *(14,15,24)* lesions had at least one PTEN mutation. KRAS mutations *(13)* were observed in 21% (six of 29) of the cancers, and all (six of six) of these also had PTEN mutations in at least one exon.

Our observations have demonstrated that complete inactivation of PTEN occurs in the great majority of endometrial carcinomas, especially those of the endo-

metrioid subtype, and even in half of all precancers (EIN). Nearly all (97%) of the endometrial cancer tissue samples that we tested had either complete absence of PTEN protein expression or reduced expression of PTEN protein (Table 1). Inactivation could be a result of structural changes (mutation or LOH) or epigenetic modification of the PTEN gene itself or its regulatory elements. Although relatively few endometrial carcinomas had biallelic structural alterations (either two or more PTEN mutations affecting both alleles or PTEN mutation in one allele and LOH of the other), we found complete loss of PTEN protein expression in 61% (20 of 33) (Table 1). Although the distribution of multiple exonic hits between one or two alleles is unknown, the number of PTEN exons affected by mutation provides some indication of that fraction of cases that are candidates for biallelic mutational inactivation.

Morphometrically defined precancers are usually diagnosed as atypical endometrial hyperplasias. It is, therefore, of interest to note that, among computerized morphometrically diagnosed precancers in which approximately half had PTEN mutations, three quarters displayed complete absence of PTEN protein (Table 1). Although only nine computerized morphometry-defined indeterminate endometrial samples were available for analysis, more than half (56%) showed no PTEN protein expression. In contrast, only one of 20 morphometrically diagnosed normal tissues did not express PTEN protein.

PTEN is a major gene involved in the pathogenesis of endometrioid endometrial adenocarcinoma. Our data suggest that altered PTEN function is partly responsible for the etiology of the majority of endometrial cancers with a premalignant phase and participates in their progression to carcinoma. Thus, decreased PTEN expression or function is a marker of the earliest endometrial precancers, and we propose that use of PTEN immunostaining in a clinical setting may be informative in identifying premalignant lesions that are likely to progress to carcinoma.

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Postdoctoral Training:

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1988-89	Intern, Internal Medicine, Beth Israel Hospital, Boston, MA
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Licensure and Certification:

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Academic Appointments

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1995	Scientific Steering Subcommittee, Gastrointestinal Cancer Center, Dana-Farber Cancer Institute, Boston, MA
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National

1996-	Reviewer, Department of Veterans Affairs Merit Review Applications
1997-98	Reviewer and Expert Consultant, American Society of Clinical Oncology Task Force on Cancer Genetics Education
1998-	Reviewer, Molecular Biology 3 Study Section, Department of Defence US Army Research Medical and Material Command Breast Cancer Research Program
1999	Reviewer, Susan G. Komen Breast Cancer Research Foundation Grants
1999	Site Visit Team Member, Quadriannual Site Visit, National Institute of Child Health and Development, Developmental Endocrinology Branch
1999	Reviewer, Cancer Genetics Section, American Society of Human Genetics Annual Meeting Abstracts
1999-	National Comprehensive Cancer Network (NCCN) Guidelines Panel Member: Genetics/Familial High Risk Screening Guidelines
2000-	Ad hoc study section member, Mary Kay Ash Charitable Foundation Women's Cancer Research Grants
2000-	Clinical Cancer Genetics Proficiency Examination Board, Institute for Clinical Evaluation, Philadelphia

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International

1994- Coordinator and co-chair, International *RET* Mutation Consortium
1994- Coordinator and chair, International Cowden Syndrome Consortium
1995-98 International Review Board, Dutch Cancer Society
1997 Ad Hoc Review Committee, Programme Project Grant, National Cancer Institute of Canada
1997- Peer Review Panel, Project Grants, Comitato Promotore Telethon, Italy
1997- Reviewer, Project Grants and Clinical Research Fellowships, Cancer Research Campaign, London, UK
1997-99 Reviewer and Full Member, National Cancer Institute of Canada, Panel J: Pathology, Tumor Markers, Molecular Epidemiology and Clinical Correlative Studies, Toronto, ON
1998- Ad Hoc External Reviewer, Italian Association for Cancer Research
1998- Member, Steering Committee, Breast Cancer Information Core (BIC)
1999- Member, International Scientific Committee, 8th International Workshop on Multiple Endocrine Neoplasia, Jerusalem, Israel, May 2001
1999 Ad Hoc Reviewer, Joint Infrastructure Fund (JIF) applications, Wellcome Trust, London, UK
2000 External Reviewer, Medical Research Council of Canada

Professional Societies, Honor Societies and Colleges:

1982- Phi Beta Kappa, Member
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1995- New York Academy of Sciences, Member
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Editorial Boards:

Editorships:

1998- Journal of Medical Genetics, North American Editor
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Editorial Board:

1998-	Journal of Endocrine Genetics, Editorial Board Member
2000-	Thyroid, Editorial Board Member
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Ad hoc Reviewer for:

1998-	American Journal of Human Genetics
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1997	American Journal of Surgical Pathology
2000-	Biochim Biophys Acta
1999-	BioTechniques
1997-	Blood
1998-	British Journal of Cancer
1998-	Cancer
1996	Cancer Epidemiology, Biomarkers and Prevention
1997-	Cancer Research
1997-	Carcinogenesis
1998-	Clinical Cancer Research
1995-	Clinical Endocrinology
1995-	Clinical Genetics
1999-	Endocrine
1999-	Endocrine-Related Cancer
1996-	European Journal of Endocrinology
1997-	European Journal of Human Genetics
1997-	Experimental Cell Research
1996-	Gastroenterology
1995-	Genes, Chromosomes and Cancer
1998-	Genomics
1997-	Human Genetics
1994-	Human Molecular Genetics
1994-	Human Mutation
1998-	International Journal of Cancer
1996-	Journal of the American Medical Association
1995-	Journal of Clinical Endocrinology and Metabolism
1999-	Journal of Experimental Medicine
1999-	Journal of Clinical Investigation
1995-	Journal of Clinical Oncology
1995-98	Journal of Medical Genetics
1998-	Journal of the National Cancer Institute
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1996	Mutation Research
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1982	Sigma Xi Certificate of Merit for Excellence in Undergraduate Scientific Research, University of Chicago, IL
1982-83	Dean's Letter of Commendation for Excellence in Gross Anatomy and Microbiology, Pritzker School of Medicine, University of Chicago, IL
1982-84	Far East Scholarship, Pritzker School of Medicine, University of Chicago, IL
1983	National Institutes of Health Summer Research Fellowship, Pritzker School of Medicine, University of Chicago, IL
1984-86	American Heart Association - Borg-Warner Medical Student Research Fellowship, University of Chicago, IL
1987	Sigma Xi, promotion to Full Membership
1988	Alpha Omega Alpha
1990	Nomination for Chief Residency, Department of Medicine, Beth Israel Hospital, Boston, for 1992-93 (Position Declined)
1990	Nomination for the National Institutes of Health-Upjohn Medical Residents Research Award
1991	Upjohn Travel Award to the Meeting of the American Association for Cancer Research, Houston, TX
1992	Johanna Wood Fellowship, Dana-Farber Cancer Institute, Boston, MA
1992-95	Cancer Research Campaign - Dana-Farber Cancer Institute Fellowship in Human Cancer Genetics, University of Cambridge, U.K.
1995-97	Lucille P. Markey Charitable Trust Young Scientist Award
1995-98	The First Lawrence and Susan Marx Investigatorship in Human Cancer Genetics, Dana-Farber Cancer Institute, Boston, MA
1996	Patterson Fellowship, Dana-Farber Cancer Institute, Boston, MA
1997-99	Barr Investigatorship, Dana-Farber Cancer Institute, Boston, MA
1999	International Scientific Committee, 8th International Workshop on Multiple Endocrine Neoplasia, Jerusalem, Israel, May, 2001
1999	Promotion to Fellowship, American College of Physicians

Laboratory and Clinical Investigator Track

A. Report of Research

1. Major research interests:

1. Cancer Genetics
2. Molecular Epidemiology of Cancer
3. Genetics of Multiple Endocrine Neoplasia Type 2 and Related Cancers
4. Familial Gastrointestinal Cancers
5. Cowden Syndrome and Related Cancers
6. Inherited Hamartoma-Neoplasia Syndromes

2. Narrative description of research

The broad thrust of my laboratory involves the utilisation of DNA-based methods to identify and characterise genes which cause susceptibility to inherited cancer syndromes, to determine their role in sporadic carcinogenesis and to perform molecular epidemiologic analyses as they might relate to future clinical applications. Upon this framework, we are examining the genetics of two inherited thyroid cancer syndromes, Cowden syndrome (nonmedullary thyroid cancer) and MEN 2 (medullary thyroid cancer), and related sporadic cancers. Hence, the genetics of susceptibility gene *PTEN*, encoding a dual specificity phosphatasw on 10q23.3, is being examined in Cowden syndrome and other inherited hamartoma syndromes as well as populations of isolated breast and thyroid cancer cases. Somatic genetics of *PTEN* is being pursued in a range of sporadic cancers including sporadic counterpart Cowden component tumors, breast, thyroid and endometrial carcinomas. Gene-gene interactions and gene-environment interactions are beginning to be explored. Biochemical, cellular and functional studies are beginning to be performed in our laboratory as well as in collaboration with a number of laboratories locally, nationally and internationally. The genetics of the *RET* proto-oncogene are pursued for clinical translational purposes for MEN 2 and sporadic neuroendocrine tumors. Towards those ends, genotype-phenotype analyses and genotype-prognosis analyses are being performed. Examination of common low penetrance variants in sporadic medullary thyroid carcinoma is also being pursued in the hope of identifying common alleles for predisposition in sporadic neuroendocrine tumors.

Recent efforts in my laboratory have focused on the role of the nuclear receptor transcription factor PPARγ in sporadic carcinogenesis. Troglitazone (Rezulin™), which is a specific synthetic ligand for PPARγ, is an oral hypoglemic agent used by over 1.6 million Americans. So, our work may have broad implications not only for examining the pathogenesis of common cancers but may impact public health as well. This avenue of investigation also promises direct translation into clinical oncologic practice.

3. Research funding information:

1981	Edmondson Summer Research Fellowship, University of Chicago (Advisor: Edward D. Garber)	PI
1978-82	Yim Chan Merit Scholarship, University of Chicago, IL	
1984-86	American Heart Association Borg-Warner Medical Student Research Fellowship, University of Chicago Pritzker School of Medicine, IL	PI
1992-95	Cancer Research Campaign [CRC] Dana-Farber Fellowship Integrated fellowship in clinical cancer genetics and molecular cancer genetics at the University of Cambridge, UK	PI

(Advisor: Bruce A. J. Ponder)

1995-97	New Investigator Award, Charles A. Dana Foundation	
1995-97	New Investigator Award, Markey Charitable Trust	
1995-98	Lawrence and Susan Marx Investigatorship in Human Cancer Genetics	PI
1996	Patterson Fellowship	PI
1996-98	Harvard Nathan Shock Center Award for the Basic Biology of Aging, NIA State of the art resource core for two dimensional gene scanning	
1996-99	Barr Investigatorship Human cancer genetics research	PI
1997-98	Women's Cancer Program Grant, Dana-Farber Partners Cancer Center Development of a rapid multi-gene test for hereditary breast cancer	PI
1997-99	American Cancer Society (National) Research Project Grant Isolation and characterisation of Cowden syndrome gene	PI
1997-1999	DFG Training Fellowship (Germany) Trainee PI: Oliver Gimm, MD Novel mutations and low penetrance alleles in the *RET* proto-oncogene in multiple endocrine neoplasia type 2 and sporadic medullary thyroid carcinoma	Mentor
1997-2000	Susan G. Komen Breast Cancer Foundation Postdoctoral Fellowship Trainee: Patricia L M Dahia, MD, PhD Role of Cowden susceptibility gene in breast cancer	PI
1998	Breast Cancer Research Award, Massachusetts Department of Public Health *PTEN*, the Cowden disease gene, in patients and families with breast cancer and thyroid disease	PI
1998-99	ASCO Young Investigator Award Prognostic markers for progression of esophageal adenocarcinoma Trainee PI: Matthew H. Kulke, MD	Mentor
1998-1999	Concert for the Cure Breast Cancer Research Award Genetics of *PTEN* in Cowden syndrome and unselected breast cancer patients	PI
1999	Ohio State University Seed Grant Mapping the susceptibility gene for hereditary and sporadic Barrett esophagus and esophageal adenocarcinoma	PI
1998-2001	Department of Defence US Army Breast Cancer Research Program Genetics of *PTEN* in different forms of hereditary breast cancer	PI
1998-2001	American Cancer Society (National) Research Project Grant Genetics of *PTEN* in Cowden syndrome and sporadic breast cancer	PI
1999-2000	Ohio State University Comprehensive Cancer Center Seed Grant	co-PI

Epidemiology of *PTEN* in prostate cancer

1999-2000 Ohio State University Comprehensive Cancer Center Seed Grant co-PI
Genetics and biology of malignant melanoma

1999-2002 National Institutes of Health Workstatement (RFP)
A phase 2 study of a selective estrogen receptor modulator (LY353381) vs. Tamoxifen vs. placebo in premenopausal women with an increased risk for breast cancer

1999-2001 Mary Kay Ash Charitable Foundation Grant PI
Genetic and functional analysis of PPAR-gamma as a novel tumor suppressor locus in sporadic breast carcinoma

2000-2003 Department of Defense US Army Breast Cancer Research Program PI
A novel phosphatase gene on 10q23, *MINPP*, in hereditary and sporadic breast cancer

2000-2002 Susan G. Komen Breast Cancer Research Foundation PI
Dissecting out the bifurcation of lipid and protein phosphatase activities in PTEN-mediated growth arrest in a breast cancer model

B. Report of Teaching

Local Contributions

Medical School / School of Public Health

1985 Medical Genetics, Teaching Assistant for 100-110 second year medical students, University of Chicago Pritzker School of Medicine (Contact 5 hr/wk, Prep 5 hr/wk)

1996-98 Molecular Epidemiology, Guest Lecturer for 30-50 medical, dental and graduate students, medical fellows and instructors, Harvard School of Public Health (Contact 1-2 hr, Prep 2 hr)

1997 HMS211A Graduate Course in Biochemistry and Cell Biology, invited lecture on inherited cancer syndromes for 20 graduate, dental and medical students, Harvard Medical School, Boston: (Contact 1.5 hr, Prep 2 hr)

1998 Harvard Medical School Course in Genetics, Embryology and Reproduction, Tutor for group of 7-10 medical students (Contact 40 hr, Prep 20 hr)

Graduate Medical Course/Seminar/Invited Teaching Presentation

1991 Grand Rounds, Beth Israel Hospital, Boston: Causes of late mortality in retinoblastoma patients, invited speaker (Contact 20 min, Prep 3 hr)

1994 Department of Medicine Seminar Series, University of Cambridge School of Clinical Medicine: The many faces of *RET*, invited lecture for 50 housestaff and faculty of the Clinical School (Contact 1 hr, Prep 2 hr)

1996 Seminars in Medicine of the Beth Israel Hospital: From bench to bedside: the *RET* proto-oncogene in multiple endocrine neoplasia, invited lecture for 30-60 faculty and trainees from the Boston area (Contact 1.5 hr, Prep 3 hr)

1996 Harvard Medical School Department of Genetics Seminar: The polygenic etiology of Hirschsprung disease, invited speaker for 20-25 clinical genetics fellows, postdoctoral fellows and genetics faculty (Contact 1 hr, Prep 2 hr)

1997 Brigham and Women's Hospital Specialty Lecture for Medical Housestaff: Genetics of endocrine tumors, invited speaker for 50-60 medical housestaff (Contact 1 hr, Prep 1 hr)

1997 Massachusetts Cancer Center Seminar, Charlestown, MA: *RET*, *GDNF* and *GDNFR-α* in MEN 2, invited speaker for 30-50 PIs, postdoctoral fellows and graduate students (Contact 1.5 hr, Prep 2 hr)

1997 GI Grand Rounds, Massachusetts General Hospital: Molecular genetics of Hirschsprung disease for 15-25 GI fellows and faculty (Contact 1 hr, Prep 2 hr)

1997 Women's Cancer Program, Dana-Farber Partners Cancer Center, Boston: Identification of the Cowden syndrome susceptibility gene, invited speaker for 20-30 multidisciplinary faculty, clinical fellows, housestaff, postdoctoral fellows, graduate students (Contact 1 hr, Prep 1 hr)

1997 Breast Center Basic Biology Seminar, Dana-Farber Partners Cancer Center, Boston: Identification of the Cowden syndrome gene, a multipurpose gene which predisposes to breast and thyroid cancers, invited speaker for 40-60 multidisciplinary faculty, fellows and housestaff (Contact 1 hr, Prep 1 hr)

1997 Harvard-Longwood Seminars in the Genetics of Cancer and Aging, Boston: *PTEN* in inherited hamartoma-cancer syndromes: one gene-many syndromes? Invited speaker for 50-70 clinical and basic science faculty, postdoctoral fellows, clinical fellows, and graduate students from the Harvard Longwood area (Contact 1 hr, Prep 1 hr)

1997 Massachusetts General Hospital Cancer Center Grand Rounds, Boston: *PTEN* in Cowden syndrome and sporadic breast and thyroid cancers, inivted speaker for 30 clinical and basic science faculty, clinical fellows and residents (Contact 1 hr, Prep 1 hr)

1999 Ohio State University Human Cancer Genetics Program Seminar, Columbus, OH: *PTEN* and the great imitator: Cowden syndrome; invited speaker for 60 faculty, staff and trainees of the Program (Contact 1 hr, Prep 1 hr)

1999 Ohio State University Comprehensive Cancer Center Grand Rounds, Columbus, OH: *RET* gene testing in multiple endocrine neoplasia: Paradigm for the practice of molecular oncology; invited speaker for >100 faculty, fellows, medicine housestaff and medical students (Contact 1 hr, Prep 30 min)

2000 Ohio State University Department of Internal Medicine Didactic Lecture for Housestaff, Columbus, OH: Clinical Cancer Genetics 101: when to make that referral for 50 internal medicine housestaff (Contact 45 min, Prep 45 min)

2000 Department of Molecular Virology, Immunology and Medical Genetics, Ohio State University Seminar: Role of *PTEN* in hereditary and sporadic breast cancer for 50 faculty, postdoctoral fellows and graduate students (Contact 1 hr, Prep 30 min)

2000 Department of Veterinary Biosciences, Ohio State University Seminar: Genetic and epigenetic alterations of PTEN in sporadic neoplasia for 50 faculty, postdoctoral fellows, residents and graduate students (Contact 1 hr, Prep 1 hr)

2000 Department of Internal Medicine Grand Rounds, Ohio State University: The yin and yang of genetic testing: practice of molecular oncology in the 21st century for 150-200 faculty, fellows, housestaff and medical students (Contact 1 hr, Prep 1 hr)

2000 Division of Hematology/Oncology Teaching Seminar, Ohio State University: Clinical Cancer Genetics 101 for 16 clinical fellows and 10 faculty (Contact 1 hr, Prep 1 hr)

Continuing Medical Education Course

1997 Cancer Genetics for Office Practice: Genetics of thyroid cancer in everyday practice, faculty (Contact 3 hr, Prep 1 hr)

1997 American College of Surgeons, Massachusetts Chapter, Waltham: Genetics of colorectal tumors, faculty (Contact 2 hr, prep 1 hr)

1999- Massachusetts Eye and Ear Infirmary and Harvard Medical School Course on Thyroid and Parathyroid Tumors: *RET* and medullary thyroid carcinoma, faculty (Contact 30 min, prep 20 min)

2000 Hereditary Cancer Syndromes: Are your patients at risk? Genetic technologies and risk management in the new millenium, City of Hope National Medical Center, Duarte, CA: Multiple endocrine neoplasia and Cowden syndrome, faculty for ~180 practising physicians, nurses and genetic counselors (Contact 7 hr, prep 3 hr)

2000 Approaches to Treating Breast Cancer. State of the Art in 2000, Columbus, OH: What's new in the understanding of genes which predispose to breast cancer risk, faculty for 250 practising physicians, oncology fellows and nurses (Contact 2 hr, prep 1 hr)

Advisory and Supervisory Responsibilities

1988-89 Teaching and supervision of Harvard medical students during clinical clerkship, Beth Israel Hospital, 1 medical student per rotation (200 hr/yr)

1989-91	Teaching and supervision of Harvard medical students during clinical clerkship and medical interns, Beth Israel Hospital, 2-4 interns +/- 1 medical student per rotation (200 hr/yr)
1991-92	Teaching and supervision of medical students, and medical housestaff from Brigham and Women's Hospital and Beth Israel Hospital, 3-8 housestaff +/- 1 medical student per month (500 hr/yr)
1993-95	Teaching and supervision of technicians, students and junior postdoctoral fellows, CRC Human Cancer Genetics Research Group, Department of Pathology, University of Cambridge, 2 technicians, 0-3 medical/graduate students and 0-1 junior postdoctoral fellow (20 hr/wk)
1995-	Teaching and supervision of postdoctoral fellows, students and technicians working in my laboratory, 2-6 postdoctoral fellows, 0-1 medical students, 0-1 undergraduate students, 1-3 technicians (15 hr/wk)
1996-98	Teaching and supervision of medical oncology and genetics fellows and genetics counsellors, Cancer Risk and Prevention Clinic, Dana-Farber Cancer Institute (3-5 hr/wk)
1996-98	Clinic Attending for medical oncology fellows, Dana-Farber Cancer Institute, 1-6 fellows per session (5-10 hr/mth)
1999-	Direction and administration of the Clinical Cancer Genetics Program, Comprehensive Cancer Center, Ohio State University: 1-2 MD attending clinical cancer geneticists, 0-1 oncology fellow, 0-1 medical resident, 3-5 cancer genetics counselors, 1-2 research assistants, 1 data manager and 2 executive support associates (20 hr/wk)

Laboratory-Based Trainees

Postdoctoral Trainees

Debbie J. Marsh, PhD 1996-99
Project: Genetics of Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome
Current Position: Lecturer, Dept of Medicine, University of Sydney School of Medicine, Sydney, Australia

Matthew H. Kulke, MD 1997-99
Project: Molecular epidemiology and prognostic markers in sporadic gastrointestinal cancers
Current Position: Instructor in Medicine, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

Patricia L.M. Dahia, MD, PhD 1997-
Project: Somatic genetics and biochemical expression of *PTEN* in sporadic tumors
Current Position: Postdoctoral Senior Research Associate, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA; Instructor in Medicine, Harvard Medical School

Oliver Gimm, MD 1997-
Project: Genetics of neuroendocrine tumors
Current Position: DFG Postdoctoral Researcher, Human Cancer Genetics Program, Ohio State University, Columbus, OH

Aurel Perren, MD 1998
Project: Immunocytochemistry of PTEN in sporadic tumors of the breast and thyroid
Current Position: Resident in Pathology, University of Zürich School of Medicine, Zürich, Switzerland

Jen Jen Yeh, MD 1998-99
Project: Somatic genetics of non-medullary thyroid carcinomas and the role of the mitochondrial genome
Current Position: Senior Resident, Department of Surgery, Boston University Medical School, Boston, MA

Liang-Ping Weng, MD, MS 1998-
Project: Biochemistry and cell biology of *PTEN* in breast carcinogenesis
Current Position: Research Scientist, Human Cancer Genetics Program, Ohio State University, Columbus, OH

Xiao-Ping Zhou, MD, PhD 1998-
Project: Genetics of central nervous system and neuroendocrine tumors
Current Position: Postdoctoral Researcher, Human Cancer Genetics Program, Ohio State University, Columbus, OH

Ravshan Burikhanov, PhD 1999-2000
Project: Cell biology of RET, PTEN and PPARgamma in thyroid cancer models
Current Position: Senior Scientist, Uzbek Institute for Medical Research, Uzbekistan

Keisuke Kurose, MD, PhD 1999-
Project: Genetics of PTEN and PPARgamma in women's cancers
Current Position: Postdoctoral Researcher, Human Cancer Genetics Program, Ohio State University Columbus, OH

Mary Armanios, MD 2000
Project: Genetics of endocrine neoplasia and neuroendocrine disorders
Current Position: Medicine-Pediatrics Resident, Ohio State University College of Medicine, Columbus, OH

Margaret Ginn-Pease, PhD 2000-
Project: Diploidy to haploidy for analysis of genetic and transcriptional mechanisms of inactivation of PCR-based *PTEN* mutation negative hereditary and sporadic breast cancer cases
Current Position: Postdoctoral Researcher, Human Cancer Genetics Program, Ohio State University, Columbus, OH

Alexander Niess, MD 2000-
Project: Common low penetrance alleles in neuroendocrine tumors and neurocristopathies
Current Position: Postdoctoral Researcher, Human Cancer Genetics Program, Ohio State University, Columbus, OH

Student Trainees

Antje Gössling 1996
Project: Genetics of GDNF and GFRα-1 in central nervous system tumors
Degree obtained: MD
Current Position: Resident in Clinical Genetics, Faculty of Medicine, University of Türbingen School of Medicine, Germany

Eva-Maria Dürr 1998
Project: Genetics of *CUL2* and *VBP-1* in phaeochromocytomas
Degree obtained: MD
Current Position: Resident in Neurology, University of Bonn School of Medicine, Germany

Ben Oatis 1999-
Project: Low penetrance alleles in the genes encoding proteins complexing with RET
Current Position: Junior undergraduate student, Department of Microbiology, Ohio State University, Columbus, OH

Christopher Alvarez-Breckenridge 1999-
Project: Genetics of sporadic solid tumors and the microenvironment
Current Position: Senior student, St. Charles Preparatory School, Columbus, OH

Students' Thesis Committees
Isabelle Schuffenecker
Degree obtained: Doctorat (PhD), 1997
Thesis: Mutations germinales du gène *RET* associées aux néoplasies endocriniennes multiples de type 2
I'Université Claude Bernard – Lyon I, Lyon, France
Role: External research advisor (1996-97) and Rapporteur (external examiner) [Gilbert Lenoir, DVM, PhD, Advisor and Chair]
Current Position: Postdoctoral Fellow, Molecular Virology, I'Université Claude Bernard – Lyon I, Lyon, France

Filip Farnebo, MD
Degree obtained: PhD, 1998
Thesis: Molecular mechanisms of tumor development in hyperparathyroidism
Karolinska Institute, Stockholm, Sweden
Role: Faculty Opponent (External examiner) [Catharina Larsson, MD, PhD, Advisor]
Current Position: Research Associate, Boston Children's Hospital and Harvard Medical School, Boston, MA

Pär-Johan Svensson, MD
Degree obtained: PhD, 1999
Thesis: Molecular studies on Hirschsprung disease and "Ondine's curse"
Karolinska Institute, Stockholm, Sweden
Role: External research advisor (1994-99) [Agnetha Nordenskjöld, MD, PhD, and Maria Amvret, PhD, Advisors]
Current Position: Pediatric Surgery Resident, Karolinska Hospital, Stockholm, Sweden

Ying Huang 1999-
Project: Mapping the susceptibility gene for familial nonmedullary thyroid cancer
The Ohio State University, Columbus
Role: PhD thesis committee member (Albert de la Chapelle, MD, PhD, Advisor and Chair)

Anu-Maria Loukola, MS 2000
Project: Molecular diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC)
University of Helsinki, Finland
Role: External examiner (Lauri A. Aaltonen, MD, PhD, Advisor)

Awards to Trainees

1997	Oliver Gimm, MD	DFG Fellowship (Germany) 1997-99
1998	Matthew H. Kulke, MD	ASCO Young Investigator Award 1989-99
1999	Patricia Dahia, MD, PhD	Election to Membership, Sigma Xi Scientific Honor Society
1999	Jen Jen Yeh, MD	Best Resident Abstract Award, New England Cancer Society
2000	Oliver Gimm, MD	Nomination to Membership, Sigma Xi Scientific Honor Society

2000 Jen Jen Yeh, MD Nomination to Membership, Sigma Xi Scientific Honor Society

Junior Faculty Mentored

Matthew H. Kulke, MD Instructor in Medicine, Dana-Farber Cancer Institute
ASCO Young Investigator Award 1998-99

Kornelia Polyak, MD, PhD Assistant Professor of Medicine, Dana-Farber Cancer Institute
ASCO Career Development Award 1999-2003

Patricia L M Dahia, MD, PhD Instructor in Medicine, Dana-Farber Cancer Institute 1999-

Liang-Ping Weng, MD, MS Research Scientist, Ohio State University 1999-

Leadership Role

1995-99 Director, Harvard Longwood Seminars in the Genetics of Cancer and Aging, organisation and coordination of seminar topic and speakers, invitation of speakers, and public relations for the seminar (CME 1 course)

1999- Director, Clinical Cancer Genetics Program, Comprehensive Cancer Center, Ohio State University

Regional, National and International Contributions (Invited Presentations)

1993 Lancet Grand Round: Familial Cancer Syndromes. Case Presentations and Multiple Endocrine Neoplasia Type 2A, Royal Marsden Hospital, Sutton

1993 ICRF Department of Medical Oncology Seminar, St. Bartholomew's Hospital, London: The multiple endocrine neoplasia type 2 syndromes

1994 Faculty, March of Dimes 25th Clinical Genetics Conference, Orlando, FL, USA Symposium in Genetics and Development: The molecular genetics of multiple endocrine neoplasia type 2

1994 Arbeitsgemeinschaft für Gynäkologische Onkologie, Vienna, Austria: The familial and genetic risks of ovarian cancer

1994 Postgraduate Training Course in Endocrinology: Multiple Endocrine Neoplasia Type 2. British Society for Endocrinology, St. Mary's Hospital, London, UK

1994 Symposium on Genotype-Phenotype Correlations, British Medical Genetics Conference, York, UK: Mutations of the *RET* proto-oncogene in the multiple endocrine neoplasia type 2 syndromes and Hirschsprung disease

1995 Case Presentation Conference, Department of Medical Genetics, BC Children's Hospital, University of British Columbia, Vancouver: The role of the *RET* proto-oncogene in the multiple endocrine neoplasia type 2 syndromes and Hirschsprung disease

1995 Meeting of the Clinical Molecular Genetics Society, Selwyn College, Cambridge, UK: Mutational analysis of the *RET* proto-oncogene in MEN 2

1995 Department of Internal Medicine IV - Nephrology Special Seminar, Albert Ludwigs University of Freiburg, Germany: Phaeochromocytoma and multiple endocrine neoplasia type 2: molecular genetic analysis

1995 EORTC Thyroid Group Meeting, London, UK: Germline mutations in the *RET* proto-oncogene in the multiple endocrine neoplasia type 2 syndromes

1995 Wessex Regional Genetics Laboratory Seminar, Salisbury, UK: The many faces of *RET*: multiple endocrine neoplasia type 2 and Hirschsprung disease

1996 Journées Internationales H P Klotz d'Endocrinologie Clinique, Paris, France: *RET* mutations in multiple endocrine neoplasia type 2 and sporadic medullary thyroid carcinoma

1996 Special Seminar, Institut Curie, Paris, France: Mapping of the Cowden disease susceptibility gene: clue to *BRCA3*?

1996 Medical Genetics Seminar, Institut Necker, Hopital des Enfants-Malades, Paris, France: Mutations in the *RET* proto-oncogene in MEN 2 and Hirschsprung disease

1996 Department of Endocrinology Seminar, King's College Hospital School of Medicine, London, UK: *RET* proto-oncogene in MEN 2 and sporadic MTC

1996 Department of Endocrinology Seminar, St. Bartholomew's Hospital, London, UK: Localisation of the gene for Cowden disease: another breast cancer susceptiblity gene?

1996 Special Seminar, Department of Medical Genetics, Queen's University, Kingston, ON: Cowden syndrome

1997 Université Claude Bernard Lyon I, Lyon, France: External examiner, PhD thesis committee (PhD Candidate: Isabelle Schuffenecker)

1997 Special Seminar, International Agency for Research on Cancer, Lyon, France: Molecular genetics of Cowden syndrome

1997 Special Seminar, Cancer Institute of New Jersey, New Brunswick, NJ: *PTEN* in Cowden syndrome

1997 31st Patterson Symposium: Li-Fraumeni syndrome, Manchester, UK: Two-dimensional gene scanning for rapid *p53* mutation detection

1997 IV International Thyroid and Neuroendocrine Cancer Workshop, Sicily, Italy: Genotype-phenotype correlations in MEN 2 and genotype-prognosis studies in sporadic medullary thyroid carcinoma

1998 Special Seminar, Fox Chase Cancer Center, Philadelphia: *PTEN*, encoding a dual specificity phosphatase, in inherited hamartoma-tumor syndromes

1998 Endocrine Grand Rounds, Mt. Sinai Medical Center, NY: The *RET* proto-oncogene in inherited and sporadic medullary thyroid carcinoma

1998 Special Seminar, Human Cancer Genetics Program, Comprehensive Cancer Center, Ohio State University, Columbus, OH: The paradox of the *RET* proto-oncogene: multiple endocrine neoplasia and Hirschsprung disease

1998 Special Seminar, Human Cancer Genetics Program, MD Anderson Cancer Center, Houston, TX: *PTEN* in inherited hamartoma-tumour syndromes

1998 Invited Lecture, First International Lentigenosis Meeting, National Institutes of Health, Bethesda, MD: *PTEN*, Cowden syndrome and Bannayan-Ruvalcaba-Riley syndrome

1998 Invited Symposium Lecture, Fourth European Congress of Endocrinology, Seville, Spain: *RET* and *PTEN* mutations in sporadic thyroid tumours

1998 Invited Lecture, ASCO Continuing Medical Education Course "Cancer Genetics in Office Practice," Princeton, NJ: Genetics of colorectal cancer

1998 Breast Cancer Research Centre, Vancouver, BC: *PTEN* and its role in breast tumourigenesis in Cowden syndrome

1998 Invited Lecture, 54th Recent Progress in Hormone Research, Skamania Lodge, Stevenson, WA: *PTEN*, encoding a phosphatase, in hereditary and sporadic nonmedullary thyroid tumors

1998 Invited Lecture, Gordon Research Conference DNA Alterations in Transformed Cells: New insights into the molecular genetics of cancer, Colby-Sawyer College, NH: *PTEN* mutations in two inherited hamartoma-cancer syndromes and sporadic tumors

1998	Invited Lecture, International Congress on Hereditary Cancer Diseases, Düsseldorf, Germany: Cowden syndrome: update on genetic mechanisms and clinical features
1998	Grand Rounds, University of Michigan Cancer Center, Ann Arbor, MI: The yin and yang of inherited thyroid cancer
1998	Invited Lecture, American Psychological Association Conference on Behavioral Science and Genetics, Tyson's Corner, VA: Genetic testing: from technology to treatment
1998	Karolinska Institute, Stockholm, Sweden: Faculty Opponent for PhD Thesis Defence (PhD Candidate: Filip Farnebo)
1999	Grand Rounds, NIDDK, NIH, Bethesda, MD: Genetic and epigenetic *PTEN* alterations in inherited and sporadic neoplasia
1999	Invited Lectures, NIH-sponsored Phakomatosis Revisited Workshop Rockville, MD: Hamartoses; Cowden syndrome and *PTEN*
1999	Invited Lecture, ASCO Train the Trainer Update: Bringing Cancer Genetics to Office Practice, New Orleans, LA: Molecular diagnosis of the inherited harmatoma tumor syndromes
1999	Medicine Grand Rounds, Rush Medical School, Chicago, IL: Molecular genetics in office practice: *RET* proto-oncogene mutations in multiple endocrine neoplasia type 2
1999	Molecular Medicine Program Seminar, University of Toronto, Canada: Genetics of *PTEN* in inherited and sporadic cancers
1999	Invited Symposium Lecture, American Gastroenterological Association, Orlando, FL: Feast or famine: *RET* proto-oncogene in intestinal ganglioneuromatosis and Hirschsprung disease
1999	Invited Symposium Lecture, Seventh International Workshop on Multiple Endocrine Neoplasia, Gubbio, Italy: MEN 2 and the practice of molecular oncology
1999	Invited Symposium Lecture, Seventh International Workshop on Multiple Endocrine Neoplasia, Gubbio, Italy: The role of *PTEN* in Cowden syndrome and multiple sporadic cancers
1999	Invited Plenary Lecture, Neuropathology at the Turn of the Millenium, Bonn, Germany: Will the real Cowden syndrome please stand up?
1999	Invited Lecture, Human Genetics and Genomic Biology Program, Hospital for Sick Children, Toronto, Canada: The highs and lows of *RET*: a question of penetrance in neuroendocrine neoplasia and Hirschsprung disease
2000-	Invited Lecture, Li-Fraumeni syndrome: A 30th Anniversary Celebration; An International Workshop on Advances in Cancer Genetics, Hospital for Sick Children, Toronto, Canada: PTEN and breast cancer: genetics, cell cycle arrest and apoptosis – from soup to nuts
2000	Invited Lecture, Clinical Cancer Genetics Program and Division of Molecular Medicine, City of Hope National Medical Center, Duarte, CA: The functional role of PTEN in hereditary and sporadic breast cancer
2000	Grand Rounds, Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL: The role of PTEN in hereditary and sporadic breast cancer
2000	Grand Rounds, Mt. Sinai School of Medicine Division of Medical Oncology and Cancer Center Grand Rounds, New York: *RET* gene testing in MEN 2: practice of molecular oncology
2000	Human Genetics Seminar Series, Department of Human Genetics, University of Pittsburgh, PA: Genetics of *RET* in endocrine neoplasia and Hirschsprung disease: feast or famine

C. Short Report of Clinical Activities

Description of Clinical Practice: Clinical cancer genetics; medical oncology, especially inherited hamartoma tumor syndromes, and endocrine tumors in a teaching hospital setting.

Patient Load: 20% effort in the practice of clinical cancer genetics. Patients/families seen in cancer genetics clinic are usually complex and labor intensive.

Clinical Contributions: When we and other groups discovered that germline mutations in the *RET* proto-oncogene are associated with MEN 2, clinical diagnostic testing became available within 6 months of our publication. Since then, our work as well as others' work have bourne out initial data, such that *RET* testing has now become the clinical standard of care in MEN 2 and all cases of medullary thyroid cancer. Mutation status is important in these entities because it alters clinical management for the patient and his/her family. I have also worked with at least two CLIA-certified laboratory to ensure quality control and have worked with at least one third party insurer so that *RET* testing is covered 100%.

Bibliography

Original Reports:

1. Garber ED, **Eng C**, Puscheck EE, Weil MK, Ward S. Genetics of Ustilago violacea. XII. Half-tetrad analysis and double selection. Bot Gaz 1982; 143:524-9.

2. **Eng CEL**, Strom CM. Analysis of three restriction fragment length polymorphisms in the human type II procollagen gene. Am J Hum Genet 1985; 37:719-732, 1986; 39:122.

3. **Eng CEL**, Strom CM. New syndrome: familial proportionate short stature, intrauterine growth retardation, and recurrent locking of the fingers. Am J Med Genet 1987; 26:217-20.

4. Garber ED, **Eng C**, Stevens DM. Genetics of Ustilago violacea. XXI. Centromere-linkage values and pericentric gene clustering. Curr Genet 1987; 12:555-60.

5. **Eng C**, Aronson MD. Pneumococcal bacteremia in two immunocompetent adults with otitis media and bronchitis. Lancet 1990; 336:1266 / 1991; 337:240-1 (response to critique).

6. **Eng C**, Chopra S. Acute renal failure in nonfulminant hepatitis A. J Clin Gastroenterol 1990; 12:717-8.

7. Banitt P, **Eng C**. Radiculopathy in an elderly woman. Hosp Pract 1991; 26:40.

8. **Eng C**, Korzenik J. Angina pectoris associated with 5-fluorouracil. Hosp Phys 1991; 27:54-7.

9. **Eng C**. Thoracic adenopathy: metastatic seminoma or sarcoid? Hosp Pract 1992; 27:208-10.

10. **Eng C**, Farraye FA, Shulman LN, Peppercorn MA, Krauss CM, Connors JM, Stone RM. The association between myelodysplastic syndromes and Crohn disease. Ann Intern Med 1992; 117:661-2.

11. **Eng C**, Cunningham D, Quade BJ, Schwamm L, Kantoff P, Skarin AT. Meningeal carcinomatosis from transitional cell carcinoma of the bladder. Cancer 1993; 72:553-7.

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